BACTERIOLOGICAL REVIEWS

VOLUME 10

BALTIMORE, MD
1946

CONTENTS

ACTION OF MICROÖRGANISMS ON HYDROCARBONS[1]

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CONTENTS

Many microörganisms possess the ability of utilizing hydrocarbons as a sole source of energy in their metabolism Gaseous, liquid and solid hydrocarbons in the aliphatic, olefinic and naphthenic series are susceptible to microbial decomposition Nearly a hundred species of bacteria, yeasts and molds representing thirty genera have been described which attack one or more kinds of hydrocarbons Such microbes appear to be quite widely and abundantly distributed in nature where they may be of considerable importance in the carbon cycle and to various industries

Wherever exposed to mineral solutions in which microbial life is possible, petroleum, rubber or other types of hydrocarbons may be slowly decomposed by microörganisms The microbial oxidation of hydrocarbons may help to account for the rapid disappearance of petroleum which pollutes fields and waterways, for the deterioration of certain rubber products both natural and synthetic, for the spoilage of cooling oils, for the depreciation of oiled or asphalt-surfaced highways and for the modification of petroleum or its products stored in the presence of water The failure of underground pipe-lines or electrical conduits "protected" from corrosion by paraffin-impregnated materials, elastomers or other hydrocarbon derivatives may be attributed in part to the activities of microorganisms which decompose hydrocarbons

Obtaining intermediate products of economic value such as fatty acids, for example, from the microbial decomposition of hydrocarbons, or employing micro-

[1] Contribution from the Scripps Institution of Oceanography, New Series No 295 This report represents part of the activities of Research Project 43A sponsored by the American Petroleum Institute

organisms for the elimination of industrial wastes around refineries are almost unexplored possibilities In this category, too, are certain methods of prospecting for petroleum which are based upon the ability of bacteria to utilize petroleum hydrocarbons Likewise very little is known regarding the effect of the growth of hydrocarbon-oxidizing bacteria in pharmaceutical and medicinal preparations containing petrolatum or mineral oil

These are some of the problems which are discussed on the following pages in summarizing our knowledge on the occurrence, characteristics, biochemical activities and possible economic importance of microörganisms which attack hydrocarbons

Historical Although paraffin wax is often considered to be biologically inert, Miyoshi (93) reported fifty years ago that thin layers of paraffin were penetrated by *Botrytis cinerea* The ability of fungi to attack paraffin was reported in 1906 by Rahn (104), who found that various soil molds, including *Penicillium glaucum*, decomposed paraffin and utilized it as a sole source of energy Bacteria were also found developing on the paraffin Söhngen (121) showed that paraffin was attacked by 17 different species of soil bacteria, paraffin wax being more readily attacked than petroleum ether, paraffin oil, crude petroleum or caoutchouc The widespread occurrence of microörganisms which attack paraffin is attested by many observers (cf , (53, 60, 138, 142, 145, 150, 181))

The utilization of methane by mixed cultures of soil bacteria was reported by Kaserer (83) in 1906 The bacteria also utilized hydrogen as a source of energy The microbial utilization of methane was studied more thoroughly by Söhngen (119), who noted the disappearance of methane, the production of CO_2 and the accumulation of organic matter (bacterial cell substance) in a mineral salts solution saturated under pressure with methane and oxygen From enrichment cultures Söhngen isolated a rod-shaped organism which he called *Bacillus methanicus*, now known as *Methanomonas methanica* (12) Subsequently the bacterial oxidation of methane was studied by many others (1, 43, 44, 64, 65, 94, 95, 120, 131, 151, 169, 178)

Störmer (131) was the first to demonstrate the microbial assimilation of aromatic hydrocarbons He isolated *Bacillus hexacarbovorum*, an organism capable of utilizing toluene and xylene Wagner (164) described *Bacterium benzoli a* and *b* which utilized toluene, xylene, benzene and various aliphatic hydrocarbons *B benzoli a*, which resembled *Mycobacterium phlei*, grew on phenol and pyrocatechol as well as on benzene *B benzoli b* was similar to *Mycobacterium lacticola* Mixed cultures of soil bacteria studied by Wagner quantitatively destroyed samples of crude oil which probably contained naphthenic, aliphatic and aromatic hydrocarbons

The bacteria studied by Tausz and Peter (152) failed to attack benzene, toluene or xylene, but several different aliphatic, olefinic and naphthenic hydrocarbons were attacked Caprylene, hexadecene (cetene), cyclohexane, methylcyclohexane and paraffinic hydrocarbons ranging from *n*-hexane to tetratriacontane, $C_{34}H_{70}$, were oxidized by *Bacterium aliphaticum liquefaciens* After noting a high degree of specificity in the ability of various bacteria to attack different

kinds of hydrocarbons, Tausz and Peter proposed the use of bacteria for the analysis of petroleum and for the purification of hydrocarbons

Some of the literature on the microbial utilization of petroleum hydrocarbons has been summarized in short reviews by Hessel (67), Tausson (144), Bushnell and Haas (19), Strawinski (132) and ZoBell *et al* (181) The literature on the microbial utilization of rubber hydrocarbons has been summarized by ZoBell and Beckwith (179)

MINERAL REQUIREMENTS OF HYDROCARBON OXIDIZERS

The microbial oxidation of hydrocarbons takes place in very simple media and throughout a wide range of environmental conditions When enriched with hydrocarbons the water from lakes, rivers, wells and oceans generally provides for the multiplication and biochemical activities of hydrocarbon oxidizers The conditions necessary for the microbial decomposition of oils have been summarized by Tausson (141) as follows (a) a physiologically balanced mineral salts solution, (b) the presence of a nitrogen source, such as ammonium or nitrate ion, (c) free oxygen, and (d) a reaction near neutrality

Hydrocarbon oxidizers have been cultivated in both solid and liquid media over a wide range of salinity, oxygen tension and temperature Except for the special techniques required for dissolving or dispersing gaseous, liquid and solid hydrocarbons in nutrient media, hydrocarbon oxidizers are cultivated in the same way, in general, as other types of bacteria, yeasts or molds

The mineral solution first employed by Söhngen (119) for the cultivation of methane-oxidizing bacteria consisted of 0 05% K_2HPO_4, 0 1% $MgNH_4PO_4$ $6H_2O$ and 0 01% $CaSO_4$ dissolved in distilled water Wagner's (164) mineral solution contained 0 1% each of K_2HPO_4 and NH_4NO_3, 0 025% $MgSO_4$ and traces of $FeCl_3$ and $CaCl_2$ According to Bushnell and Haas (19), the following mineral solution proved to be quite satisfactory for the cultivation of a large variety of hydrocarbon-oxidizing microörganisms

Distilled water	1,000 0 ml
$MgSO_4$	0 2 g
$CaCl_2$	0 02 g
KH_2PO_4	1 0 g
K_2HPO_4	1 0 g
NH_4NO_3 or $(NH_4)_2SO_4$	1 0 g
$FeCl_3$ (conc sol)	2 drops

A similar mineral solution was used by Stone *et al* (129) except for the addition of traces of Mn and Zn, which were thought to promote the growth of hydrocarbon oxidizers

The nitrogen requirements of most hydrocarbon oxidizers are satisfied by ammonium salts Nitrate nitrogen is utilized by many Methane-oxidizing bacteria were found (94) to grow almost equally well on any concentration of either NH_4Cl or KNO_3 ranging from 0 00025 to 0 3% Organic compounds such as leucine, asparagine or peptone also satisfied the nitrogen requirements Growth

was best in a medium containing from 0 1 to 0 5% K_2HPO_4 and very poor in media containing less than 0 005% K_2HPO_4

Bushnell and Haas (19) found that the presence of 50 mg/L of phosphate was necessary to secure minimum growth of cultures of *Pseudomonas* in media containing hydrocarbons as the only source of energy, suggesting that under certain conditions phosphorylation may play an important rôle in hydrocarbon metabolism as it does in carbohydrate metabolism The ability of *Corynebacterium* species to attack hydrocarbons was not affected by the lack of phosphate (19) We (178) have found phosphate to be beneficial for the microbial oxidation of hydrocarbons

ZoBell *et al* (181) used sea water fortified with 0 1% $FeNH_4PO_4$ for growing hydrocarbon oxidizers from marine sources Sea water diluted 1 10 with distilled water and enriched with 0 1% $FeNH_4PO_4$ proved to be an excellent mineral solution for the cultivation of hydrocarbon oxidizers from "fresh-water" sources Oil-well brines having salinities as great as 300,000 mg/L have been used successfully for the cultivation of hydrocarbon oxidizers found therein Elazari-Volcani (34) isolated bacteria from the Dead Sea which grew in 25% salt solution enriched with kerosene or petroleum Hydrocarbon oxidizers have been demonstrated (178) in marine sediments, both ancient and recent, which grew in sea water treated with 10 to 25% NaCl and enriched with paraffin oil Sea water contains an average of 3 to 3 5% solids, most of which is NaCl

Since the microbial oxidation of hydrocarbons is usually accompanied by acid production, the presence of carbonate or phosphate in the medium is desirable to buffer it at a favorable hydrogen-ion concentration The reaction may become more alkaline in hydrocarbon media in which nitrate is being reduced Tausson (144), like many earlier workers, recommended the use of media which were approximately neutral in reaction Bushnell and Haas (19), however, observed that hydrocarbon oxidizers are not extremely sensitive to changes in hydrogen-ion concentration within the range of pH 6 to 9 5 This was confirmed by ZoBell *et al* (181), who observed the luxuriant growth of hydrocarbon oxidizers throughout the range of pH 6 to 10 The assimilation of paraffin wax by *Aspergillus versicolor* was independent of the initial reaction between pH 5 8 and 7 9 (70) The hydrocarbon oxidizers studied by Strawinski (132) were active from pH 5 to 8, but they grew best at pH 7 6 to 8

EFFECT OF OXYGEN TENSION

Hydrocarbons are attacked by microorganisms growing under both aerobic and anaerobic conditions The growth of aerobes appears to be equally good at any oxygen content ranging from 0 1 to 20 or 30 mg/L (When in equilibrium with the atmosphere at 25°, the oxygen content of fresh water is approximately 8 6 mg/L) ZoBell *et al* (181) noted that the multiplication of hydrocarbon oxidizers continued unabated until the oxygen content of the medium was reduced to less than 0 1 mg/L The oxidative metabolism of such organisms, however, is influenced by the presence of oxygen (170) For example, from three to four times as much ether-extractable material was recovered (132) from aerated than

from unaerated cultures growing on naphthalene under otherwise comparable conditions Others (91, 136, 141) have also found that vigorous aeration promoted the dissimilation of hydrocarbons by microörganisms

In their pioneer work, Kaserer (83), Söhngen (119) and Störmer (131) used an atmosphere consisting of about equal volumes of oxygen and methane for demonstrating the microbial oxidation of methane Münz (94), however, found that *Methanomonas methanica* grew best in an atmosphere consisting of a higher concentration of methane than oxygen In our experience (178) about 40 volumes of oxygen to 60 volumes of other gases is about as much oxygen as can be used advantageously for the cultivation of methane oxidizers Higher concentrations of oxygen retard the multiplication of *Methanomonas methanica* Initial growth is equally good in gas mixtures containing much less than 40% oxygen, but when the initial gas mixture contains only a very little oxygen, the early depletion of oxygen arrests further activity unless the oxygen supply is replenished This may be illustrated by data from a representative experiment (178) in which methane-oxidizing bacteria were grown in a mineral solution in bottles containing 150 ml of different gas mixtures as follows

INITIAL GAS MIXTURE			CH_4 CONSUMED IN 7 DAYS AT 28°
CH_4	O_2	CO_2	
%	%	%	*ml*
20	80	0	4 7
40	60	0	12 1
60	40	0	18 3
70	30	0	20 5
80	20	0	18 9
50	45	5	32 3
50	40	10	39 4
50	40	10	38 0

The data from this and similar experiments indicate that the presence of from 5 to 10% CO_2 in the gas mixture enhances the activity of methane oxidizers Neither carbonate nor bicarbonate ion has proved to be as satisfactory as free CO_2 for initiating the growth and activity of methane oxidizers

While the microbial oxidation of hydrocarbons is primarily an aerobic process, the substances are attacked in the absence of free oxygen A good many hydrocarbon oxidizers can utilize nitrate or sulfate as hydrogen acceptor *Bacterium benzoli* (142) oxidized about 8 grams of benzene in 42 days at 28° utilizing nitrate as the hydrogen acceptor The reduction of nitrate by hydrocarbon-oxidizing microörganisms has been commonly noted (19, 138, 154, 181) The bacteria in the oil-soaked soil studied by Beckman (11) attacked hydrocarbons under either aerobic or anaerobic conditions, but the nature of the hydrogen acceptor was not specified The oxidation of petroleum by bacteria in oil-well brines under anaerobic conditions has also been reported (92)

Tausson and Aleshina (146) demonstrated the utilization of hydrocarbons by

strictly anaerobic sulfate reducers The oxidation of phenanthrene, naphthalene and related compounds by anaerobes which utilized sulfate as the hydrogen acceptor was observed by Tausson and Vesselov (149) Sulfate reducers have been observed to oxidize crude oil (89) The action of *Desulfovibrio* species on paraffinic hydrocarbons has been reported by Novelli and ZoBell (99) It is quite possible that the apparently autotrophic sulfate reducers studied by Czurda (25) were actually utilizing the paraffin wax employed to exclude oxygen from the cultures

EFFECT OF ORGANIC MATTER

The presence of peptone, carbohydrates and other types of readily utilizable organic matter interferes with the assimilation of hydrocarbons by some, but not all, microörganisms Presumably such organic materials are preferentially attacked, and their oxidation results in depletion of oxygen and lowering of the oxidation-reduction potential (175) to a point where only anaerobes can function We have been unsuccessful in demonstrating the decomposition of hydrocarbons by sulfate reducers in the presence of an abundance of other kinds of readily utilizable organic matter

Following prolonged cultivation on nutrient agar or gelatin, *Bacterium aliphaticum* attacked hydrocarbons much less readily than cultures of this organism which had been maintained on mineral salts media enriched with hydrocarbons (152) Similarly, methane oxidizers tended to lose their ability to utilize methane when cultivated in nutrient peptone or similar organic media (1) After noting its ability to grow in either organic media or in exclusively mineral media, *Methanomonas methanica* was characterized by Münz (94) as a facultative autotroph Neither 0 5% asparagine nor 1 0% peptone affected the oxidation of paraffin wax by soil microörganisms (53) It has been observed that naphthalene actually disappeared more rapidly from soil rich in organic matter than from organic-poor soils (91, 136)

Low concentrations of organic matter (less than 1 mg/L) generally promote the microbial assimilation of hydrocarbons, probably because the readily utilizable organic matter provides for the multiplication of microörganisms which then attack the hydrocarbons Moreover, the CO_2 which results from the oxidation of organic compounds may have a beneficial effect upon initiating the activity of hydrocarbon oxidizers Especially beneficial are small quantities of certain growth factors, including yeast extract, nicotinamide, riboflavin, pyridoxine, thiamine and ascorbic acid, used either alone or in combinations

After transferring their cultures 10 to 15 times on kerosene media without any diminution of growth, Bushnell and Haas (19) concluded that either hydrocarbon oxidizers do not require accessory growth factors or the organisms with which they were working were able to synthesize these substances Certain yeasts have been found to produce a growth substance resembling "bios" when grown in mineral media with paraffin as the sole source of energy (137)

Bacterium aliphaticum, which utilizes numerous paraffinic hydrocarbons, has been shown (80) to grow readily on peptone media and to utilize a large number of

carbohydrates Most of our stock cultures of hydrocarbonoclastic bacteria are maintained on peptone agar slants It has been found (178), however, that upon transferring such cultures to mineral media the bacteria attack hydrocarbons more energetically if they have been maintained on agar slants flooded with paraffin oil This suggests selective enzyme formation, although a good many stock cultures having no previous history of contact with hydrocarbons readily utilize hydrocarbons in appropriate media (58, 178)

TEMPERATURE REQUIREMENTS

Most of the experimental work on the microbial decomposition of hydrocarbons has been conducted at temperatures ranging from 25° to 37° In demonstrating methane oxidation by soil bacteria, Kaserer (83) incubated his cultures at 28° to 30° Söhngen (119) cultivated *Methanomonas methanica* at 30° to 37° Wagner (164) used an incubation temperature of 25° in studying the decomposition of hydrocarbons by *Bacillus benzoli* The methane oxidizers studied by Münz (94) were active at temperatures ranging from 18° to 40°, maximum activity occurring at 34°

Söhngen (121) was able to demonstrate the largest number of hydrocarbon-oxidizing microörganisms in soil when the cultures were incubated at 26° to 30°, but hydrocarbons were decomposed more rapidly at 37° In one series of experiments there was evidence for the microbial attack of paraffin by cultures in two days when incubated at 28° or 37°, while at 20° a week was required for such evidence Similarly, Stone *et al* (129) found hydrocarbon oxidizers to be more active at 30° and 37° than at 20°

Although an incubation temperature approximating 27° was used for studying the microbial decomposition of hydrocarbons, Haas (58) noted the growth of certain *Pseudomonas* species at 47° Some of the hydrocarbon oxidizers isolated from marine materials by ZoBell *et al* (181) decomposed seven times as much paraffin at 55° as at 22° Others were active at temperatures as low as 0°, although it required several weeks to establish this point because the rate of microbial activity at low temperatures is very slow Bacteria which destroyed phenol at 60° were studied by Egorova (33)

DISPERSION OF HYDROCARBONS IN CULTURE MEDIA

Hydrocarbons are virtually insoluble in water with the exception of gaseous ones, the solubility of which is largely a function of the partial gas pressure This presents a problem in dispersing hydrocarbons in mineral solutions so that they will be available to attacking microörganisms By maintaining an appropriate atmosphere of gaseous hydrocarbons in a closed system, it is possible to provide for an optimum concentration of such hydrocarbons in the mineral solution, but the dispersal of less soluble liquid and solid hydrocarbons requires special techniques in order to obtain the best results The chief factor which limits the microbial decomposition of hydrocarbons is the dispersion of the latter in such a way that they can be acted on by enzymes

Using a slight modification of the method employed by Söhngen (121), Bushnell

and Haas (19) noted that the vapors from slightly volatile hydrocarbons, such as petroleum ether or gasoline, poured into the lid of an inverted petri dish were sufficient to support the growth of bacteria on mineral-salts agar streaked with source material When working with relatively non-volatile hydrocarbons, such as kerosene or light oils, the hydrocarbon was poured over the surface of inoculated agar with good results Liquid cultures were covered with a thin layer of liquid hydrocarbons The thickness of the layer of hydrocarbons did not affect the growth of the microörganisms Paraffin was added to the medium in a melted condition, thereby giving a rough irregular mass of paraffin which offered sufficient surface for bacterial action Most workers have introduced paraffin wax in this manner, or else cut it into thin shavings in order to expose as much surface as possible to the action of organisms

The effect of surface area exposed to the culture is illustrated by the following data obtained in an experiment in which identical quantities of paraffin in various forms were introduced into a mineral medium (178) The medium, in 120-ml glass-stoppered bottles, was inoculated with bacteria and, as a criterion of microbial activity, the oxygen content of the medium was determined before and after incubation

SET NO	METHOD OF DISPERSION	SURFACE AREA EXPOSED TO MEDIUM	O_2 CONSUMED IN 7 DAYS AT 25
		cm^2	ml
1	One 10 mm cube	6	0 02
2	Eight 5 mm cubes	12	0 01
3	64 2 5 mm cubes	24	0 03
4	102 thin slices	208	1 32
5	Bottle surface coated	148	1 05
6	Bottle + sand surface	830	2 59
7	Bottle + glass wool	1,900	4 11
8	Control (No paraffin)	0	0 02

In the first four sets 0 9 g of paraffin (mp about 55°) was cut into pieces of different sizes and added to each bottle In the fifth set 0 9 g of paraffin was melted and the bottles manipulated while cooling so that the interior surface of the bottle was completely covered with a thin layer of paraffin. This process was repeated in dispersing the paraffin in the sixth and seventh sets of bottles whose interior surfaces were supplemented by the surface of ignited silica sand and glass wool, respectively From the data in the table it will be observed that there was a direct relation between the surface area of paraffin exposed to the culture and the consumption of oxygen ZoBell (172) has shown that bacteria in dilute nutrient solutions benefit by the presence of solid surfaces, and this is particularly true of hydrocarbon-oxidizing bacteria

Söhngen (121) found the rate of hydrocarbon utilization to be directly proportional to the surface area of culture in contact with oil, crude oil being oxidized at the rate of 15 mg per square decimeter of culture surface in 24 hours at 28°

The growth of *Micrococcus paraffinae*, *Mycobacterium album* and *Mycobacterium rubrum* on paraffin or other hydrocarbon media was largely a function of the surface of soil particles dispersing hydrocarbons in such media (122) Both silicon dioxide and iron oxide stimulated the microbial oxidation of petroleum (122) Soil microörganisms quite rapidly decomposed films of paraffin on kieselguhr and other particulate substances (53)

Recognizing the importance of the surface factor, Tausson and Shapiro (147) expressed the rate of oil oxidation in terms of surface area of oil exposed to the culture Crude oils, for example, were found to be oxidized at the rate of 250 g per square meter of free surface in seven months as compared with 100 g of cylinder oil oxidized under similar conditions

In their studies on hydrocarbon utilization by sulfate reducers, Tausson and and Aleshina (146) mixed melted paraffin with powdered glass to increase the free surface of paraffin The glass also served as a "sinker" to keep the paraffin at the bottom of the mineral medium Petroleum was introduced into the cultures in the form of a semi-liquid mass of the following composition

Calcium sulfate	20 g
Calcium carbonate	10 g
Kaolin	100 g
Petroleum	30 g

A layer of this dough-like mixture was triturated with an equal volume of mineral solution, after which a layer of the resulting homogeneous mass 8 to 10 mm thick was transferred to culture receptacles The latter were then filled with mineral solution The mass of dispersed petroleum was sufficiently stable to withstand autoclave sterilization without an appreciable separation of the petroleum from the solids (146)

In order to demonstrate (178) the presence of hydrocarbon-oxidizing sulfate reducers in soil, oil-well brines and other material, varying quantities of the material in question were introduced into test-tube "deeps" of a paste freshly prepared of the following ingredients

Plaster of Paris ($CaSO_4$)	50 00 g
Calcium carbonate	10 00 g
$FeNH_4PO_4$	0 01 g
Paraffin oil	10 00 g

These ingredients were mixed to a paste with sea water or other mineral solution Upon setting, the paste hardened into a slightly porous mass Air was excluded by a layer of paraffin wax or a mixture of paraffin and petrolatum The reduction of sulfate by hydrocarbon-oxidizing anaerobes resulted in the formation of H_2S which combined with the iron in the medium, thereby changing the color of the medium from white to black

By dispersing hydrocarbons adsorbed on the surfaces of inert solids, the microbial attack of nearly all kinds of hydrocarbons tested is greatly increased (178) Certain hydrocarbons which seemed to be invulnerable to microbial attack were

quite rapidly decomposed when dispersed throughout the medium on the surfaces of inert solids Asbestos fibers and glass wool have proved to be better than sand, presumably because the sand settles in a compact mass to the bottom of the culture medium while glass wool and asbestos, which do not pack, distribute the adsorbed hydrocarbons throughout the medium Solid particles of small dimensions such as bentonite, kaolin or talcum powder are not beneficial because they pack in a solid mass at the bottom of the culture receptable and the adsorbed oil completely fills the interstitial spaces so that relatively little surface is presented for the action of microörganisms in the mineral solution Solid particles smaller than bacteria may be injurious to them (172)

Small quantities of liquid and solid hydrocarbons can be uniformly distributed on glass wool, asbestos, sand or other inert solids by dissolving the hydrocarbon in petroleum ether or other suitable solvent The solvent is then evaporated while manipulating the bottle in such a way that all surfaces are covered

More rapid utilization is permitted by dispersing hydrocarbons by emulsification than by adsorption on solid surfaces (178) Fairly homogeneous emulsions have been prepared by mixing the hydrocarbon with the mineral solution in a Waring blender and then passing this mixture through a homogenizer several times When its addition is permissible, the use of a little gum arabic or gum acacia improves the stability of the emulsion Gum arabic is relatively inert, being attacked by very few bacteria and by these only slowly Emulsifying cetane, cetene, kerosene and paraffin oil in mineral media increased their utilization by sulfate-reducing bacteria more than tenfold as compared with their utilization when adsorbed on asbestos or glass wool

Johnson *et al* (79) isolated hydrocarbon-oxidizing bacteria from soil on plates of dilute soil-extract agar which had been emulsified with petroleum ether just before cooling

CRITERIA OF HYDROCARBON UTILIZATION

Several criteria have been used as evidence of the microbial utilization of hydrocarbons Commonest among these are the disappearance or modification of the hydrocarbon, the production of CO_2, acid formation, multiplication of microorganisms or the consumption of oxygen in media consisting of mineral solutions enriched with hydrocarbons as the only source of energy

Manometric methods have been extensively used for following hydrocarbon oxidation in a closed system, a method which is particularly applicable to quantitative experiments with gaseous hydrocarbons such as methane, ethane, propane, butane, ethylene, propylene, butylene, butadiene, acetylene and cyclopropane The culture receptacle can be fitted with a manometer to register changes in internal gas pressure, or the culture receptacle can be connected with another bottle of sterile mineral solution in such a way that the sterile solution is sucked into the culture receptacle as the hydrocarbon and oxygen are consumed The volume of oxygen consumed by hydrocarbon oxidizers always exceeds the volume of CO_2 produced

In a typical experiment Söhngen (119) observed the following changes in gas composition as a result of the activity of *Methanomonas methanica* in 102 ml of mineral medium for 14 days at 30° to 34°

	O_2	CO_2	CH_4
	ml	*ml*	*ml*
Initially present	320	0	225
Present after 14 days	172	78	0

The data indicate that part of the methane had been oxidized to CO_2 Some of the methane was converted into bacterial cell substance as indicated by the accumulation of enough organic matter in the culture medium to reduce 48 3 ml of N/10 $KMnO_4$ The accumulation of organic materials in mineral medium in which *Methanomonas methanica* was growing was also observed by Giglioli and Masoni (43)

Stone *et al* (129) used a Warburg manometric apparatus for determining the oxygen required for the dissimilation of several oils by mixed cultures It was found that the amount of oxygen consumed and CO_2 liberated varied according to the rate of shaking, the temperature of incubation and the age of the inoculum The respiratory quotients of light oils were found to be in the neighborhood of 0 63, indicating that a large percentage of the molecules attacked was completely oxidized to CO_2 The theoretical respiratory quotient for complete oxidation of a long-chain paraffin hydrocarbon with the formula C_nH_{2n+2} is approximately 0 67 (129) The fermentation of heavy oils containing longer molecules than the lighter oils did not yield as much CO_2, and the respiratory quotients were lower than for lighter oils

Bushnell and Haas (19) found the respiratory quotients of various bacterial cultures on different hydrocarbons ranged from 0 30 to 0 70, there being no direct correlation between the R Q and the nature of the hydrocarbon The R Q of washed cells of *Bacterium aliphaticum* averaged 0 47 for heptane, 0 48 for octane, 0 63 for nonane, 0 63 for dodecane and 0 88 for glucose (79)

Oxygen consumption in 60-ml glass-stoppered bottles of mineral solutions enriched with hydrocarbons as the only source of energy has been used for studying the occurrence and behavior of hydrocarbon oxidizers in marine materials (181) The same procedure has been used for studying the microbial assimilation of rubber hydrocarbons (179)

The decoloration of methylene blue by cultures of *Bacterium aliphaticum liquefaciens* growing in mineral solutions enriched with hexane signified to Tausz and Donath (151) that the dye was utilized as an hydrogen acceptor We (178) have found that while certain bacteria reduce methylene blue in the presence of certain hydrocarbons, methylene blue reduction is not a reliable criterion of hydrocarbon utilization because some bacteria which utilize hydrocarbons do not decolorize the dye

Stone *et al* (129) stated that the breakdown of oil is an oxidative process

characterized by a high bacterial count, emulsification and sometimes a decrease in pH They observed plate counts ranging from 12,000,000 to 1,470,000,000 per ml in inoculated media enriched with various crude oils or fractions thereof after four days' incubation at room temperature In the majority of cases the maximum plate counts were obtained after three to six days of incubation Thousandfold or greater increases in the bacterial population of mineral media enriched with various petroleum fractions have been observed (19) after 7 to 14 days' incubation at 27° An increase in the plate counts of soil treated with benzene and other aromatic hydrocarbons has been reported (91) Mineral media inoculated with soil and enriched with cetane, naphthalene and biphenyl have been found (133) to contain bacterial populations exceeding a billion per ml after a few days of incubation

While the appearance of turbidity in hydrocarbon media has been very commonly used as a criterion of growth of microörganisms therein, extensive multiplication may occur without rendering the medium turbid This is because some organisms are intimately associated with solid surfaces (172) or with the hydrocarbon Reed and Rice (105), for example, found that only 5 to 30% of the acid-fast organisms examined remained in the water, 70 to 95% migrated to the oil phase Tausz and Peter (152) related that, although the medium showed no turbidity, bacteria attacking the overlying oil perforated and rendered inhomogeneous layers 1 to 2 mm in thickness The undersurface of the oil layer in contact with the medium became stringy and small droplets, pitch-like in color and consistency, broke off and sank to the bottom of the culture flask In other experiments (152) the medium overlayed with oil became turbid in three days

The emulsification of various kinds of oil by microörganisms has been observed by numerous investigators (11, 19, 130, 147, 152, 178) The microbial emulsification of oil is due in part to the production of organic acids and detergents and in part to the penetration of the oil by myriads of microörganisms which cause either a mechanical or chemical disintegration of the oil Tausz (150) observed the physical disintegration of oil by motile bacteria dragging microscopic droplets of oil with them as they dart in and out of the oil overlaying the mineral solutions The production of detergents or surface-active substances by bacteria associated with oil-bearing sediments has been reported by ZoBell (173) The formation of CO_2 by bacteria tends to emulsify oil

Acid production by active cultures of hydrocarbon oxidizers is definite though usually inappreciable. Decreases of from 0 1 to 1 5 units have been observed in the pH of media in which hydrocarbons are undergoing microbial oxidation. Carbonic and organic acids produced during the dissimilation of hydrocarbons are responsible for the pH decreases

OXIDATION PRODUCTS

CO_2 always results from the microbial dissimilation of hydrocarbons Büttner (20) found that from 80 to 90% of the carbon in the paraffin decomposed by soil organisms in 21 to 31 days could be accounted for as CO_2 The remainder was

believed to be converted into cell substances, fatty acids and possibly other intermediate products Some of his (20) data are summarized below

MICROÖRGANISM	PARAFFIN DECOMPOSED	CO_2 PRODUCED	C IN PARAFFIN	C IN CO_2	C OXIDIZED TO CO_2
	mg	*mg*	*mg*	*mg*	%
Mycobacterium lacticola	224	585	192	162	84 4
Mycobacterium phlei	182	504	156	137	87 9
Mycobacterium cos (rubrum)	170	460	146	125	85 6
Pseudomonas aeruginosa	90	251	77	69	89 5
Actinomyces chromogenes	81	234	70	64	91 3

Haag (56) pointed out that the amount of paraffin oxidized which could be accounted for as CO_2 in Büttner's experiments may be somewhat too high, because the amount of paraffin decomposed was determined by difference in the amounts extractable by carbon tetrachloride, and the latter would dissolve not only the residual paraffin but also certain lipids that occur particularly abundantly in acid-fast bacteria

From data given by Söhngen (120), it is estimated that about half of the methane assimilated by *Methanomonas methanica* was oxidized to CO_2 and the other half was converted into bacterial cell substance and other products of metabolism From 20 to 25% of the hexane, C_6H_{14}, utilized by marine bacteria (181) was converted into bacterial protoplasm Similar results were obtained with tetratriacontane, $C_{34}H_{70}$

An appreciable portion of the paraffin oxidized by *Aspergillus flavus* was converted into mold mycelium (142) During the first two weeks of incubation the "economic coefficient," defined by Tausson (142) as the per cent of consumed paraffin accounted for as mold mycelium, was about 90% After six or seven weeks the "economic coefficient" ranged from 50 to 60% Assuming that the carbon content of the paraffin and mold mycelium was 85 and 45% respectively, from 25 to 45% of the carbon of the paraffin consumed was converted into mold mycelium Esters of fatty acids and higher alcohols were detected as intermediate products of metabolism An appreciable portion of the energy resulting from the oxidation of paraffin by *Aspergillus* and *Penicillium* species is lost as heat (148)

Besides producing CO_2 and cell substance, most of the 17 species of hydrocarbon-oxidizing bacteria studied by Söhngen (121) produced organic acids as intermediate products of metabolism The formation of fatty acids was believed by Tausson and Shapiro (147) to account for the marked increase in the saponification number and the emulsification of crude oil samples undergoing microbial decomposition The same view was expressed by Stone *et al* (130), who state that the drop in pH, the emulsification of unfermented oil and the appearance of a residue indicated that some long-chain organic acids were produced by microorganisms attacking petroleum Similarly, Bushnell and Haas (19) interpreted changes in pH and emulsification of oil in water as evidence that long-chain organic acids were formed during the microbial decomposition of hydrocarbons

Bushnell and Haas (19) claimed that there was also some evidence to suggest that unsaturated hydrocarbons were formed by bacteria growing on mineral oil Traces of unidentified unsaturated hydrocarbons were detected by Tausz and Donath (151) in cultures of *Bacterium aliphaticum liquefaciens* growing on hexane *Mycobacterium* species growing on paraffin wax were observed by Haag (56) to cause a decrease and then an increase in the iodine number, which was believed to indicate that the bacteria preferentially attacked double bonds and then produced unsaturated compounds

Indirect evidence was obtained by Hopkins and Chibnall (70) which suggested that ketones may be primary products resulting from the oxidation of higher paraffins by *Aspergillus versicolor* Further oxidation of the ketones resulted in the production of shorter fatty acids, although CO_2 and mold mycelium were the principal end products of metabolism

From a culture of mold resembling *Aspergillus flavus* which had been growing for 12 weeks in a medium initially containing 3 65 g of paraffin, Tausson (138) recovered 1 24 g of mold mycelium, 1 29 g of residual paraffin and 0 234 g of non-acidic products which were assumed to consist in part of esters of the higher aliphatic alcohols Fatty acids and CO_2 were produced during the oxidation of paraffin by certain yeasts (137)

The formation of a compound containing the oxyphenol group resulted from the action of sulfate reducers on phenanthrene and retene (149) Orthosalicylic acid was detected as one of the main products resulting from the oxidation of naphthalene by *Pseudomonas aeruginosa* (134) Jacobs (73) identified phthalic acid in cultures of soil bacteria dissimilating naphthalene Significant quantities of salicylic acid resulted from the dissimilation of naphthalene by the *Pseudomonas* species studied by Strawinski (132) There was some evidence that the dissimilation of naphthalene passed through the aldehyde stage, and the formation of a complex substance of higher molecular weight than naphthalene was noted The oxidation of naphthalene and cetane gave the medium an acid reaction (132)

Besides traces of acetic and lactic acids, Thaysen (155) reported the formation of methane, ethane and acetaldehyde from the microbial decomposition of kerosene

Methods for the bacterial conversion of gaseous paraffinic hydrocarbons to oxygenated organic compounds ranging from low boiling point alcohols to waxy acids, esters and alcohols have been described in the patent literature (135a) It has been claimed that under certain conditions heavier hydrocarbons such as butane are susceptible to conversion to unsaturated compounds capable of undergoing polymerization to produce heavy hydrocarbon molecules Allegedly these conversions are catalyzed by *Bacillus methanicus* and *B ethanicus*

From bacterial cultures growing on mineral oil, Haas *et al* (60) isolated small quantities of organic acids, the melting points of which ranged from 25° to 30° No sterols were detected in bacterial cultures acting on oil, but molds growing on hydrocarbons were found to produce small amounts of ergosterol and cholesterol β-Carotene was detected as an end product resulting from the growth of *Coryne-*

bacterium species on paraffin oil It was reported (59) further that two other carotenoid pigments besides β-carotene and astacin were produced by a species of *Mycobacterium* growing on a substrate composed of mineral salts and paraffin oil

A volatile alcohol, probably isopropanol, was found in a mixed culture of yeasts and molds growing on paraffin oil (58) Glycol and glycerol were produced from paraffin oil by a *Mycobacterium* species Several of the bacteria produced small quantities of oil-soluble organic acids from the oxidation of hydrocarbons The oxidation of cetane by *Pseudomonas aeruginosa* resulted in no change of pH, according to Schuman *et al*[1] (114), but the pH was materially lowered during the oxidation of naphthalene by this organism

Acid formation by *Bacterium benzoli* growing in mineral media enriched with benzene was reported by Wagner (164) CO_2 was the principal product resulting from the oxidation of benzene, although there was indirect evidence for the formation of hydroquinone and various organic acids *Bacterium benzoli* also produced acid during the oxidation of toluene

About 15% of the isotopic 7,8,9,10-tetradeuterio-*n*-hexadecane absorbed by rats was found by Stetten (127) to be oxidized to fatty acids, apparently in the liver The catabolic route of the hydrocarbon was indicated by the appearance of a significant though low concentration of D_2O in the body water and a notably higher isotope concentration in fatty acids of the carcass The hexadecane was believed to be absorbed prior to alteration of the molecule by bacteria in the intestine and to be converted into palmitic acid by the oxidative attack on a terminal methyl group El Mahdi and Channon (36), working with rats, and Channon and Devine (22), working with cats, concluded that *n*-hexadecane was absorbed and catabolized by these animals, after finding much less of the hydrocarbon in the body and excrements than the amount fed The recovery of muconic acid containing 7 2 atom% deuterium from the urine of rabbits injected with deuterio-benzene, C_6D_6, was regarded as evidence that rabbits can oxidize benzene (62a) The oxidation of *p*-cymene to cumic acid by sheep and the partial oxidation of two carcinogenic hydrocarbons, 3,4-benzopyrene and 1,2,5,6-dibenzoanthracene, by rats and mice has also been reported (62a) The modification of carcinogenic hydrocarbons by microbial enzymes is a possibility which merits consideration

KINDS OF HYDROCARBONS ATTACKED

Crude oils, illuminating gases, petroleum ethers, gasolines, kerosenes, fuel oils, paraffin or mineral oils, petrolatums, asphalts, paraffin waxes, and rubber both natural and synthetic, besides numerous chemically pure hydrocarbons have been shown to be oxidized by a great variety of microörganisms Although numerous papers on this subject have appeared during the last forty years, our knowledge is still so fragmentary that any generalizations may be premature The interpretation of experimental results is complicated by the complexity and highly variable composition of crude oils, asphalts, rubbers, etc In the face of inadequate information on the chemical composition of such substances, it is indeterminate whether observed results are attributable to particular hydro-

carbons, other types of organic compounds or inorganic constituents in the products, or to peculiar experimental conditions Even work with pure hydrocarbons is complicated by vast differences in physical properties including solubility or miscibility in water, which make it virtually impossible to test their utilizability under strictly comparable conditions

In the aliphatic series it appears that, in general and within certain limits, long-chain hydrocarbons are attacked more readily than compounds having only a few carbon atoms per molecule Substantiating this generalization is the fact that methane, CH_4, ethane, C_2H_6, and propane, C_3H_8, are oxidized by relatively few organisms and by these only slowly, while paraffin waxes consisting mainly of compounds ranging from $C_{20}H_{42}$ to $C_{40}H_{82}$ are utilized readily by a large number of microörganisms A partial list of the microörganisms which have been reported to assimilate paraffin wax is given in table 1

Söhngen (121) reported that the growth of certain cultures in 200 ml of mineral medium enriched with 2 g of finely divided paraffin wax resulted in the oxidation of the following quantities of paraffin in a month at 28°

CULTURE	PARAFFIN OXIDIZED
	mg
Mycobacterium album	300
Mycobacterium rubrum	330
Micrococcus paraffinae	180
Bacterium fluorescens liq	180
Raw culture	540

None of these pure cultures could assimilate methane, and the bacteria grew less rapidly on pentane, hexane, heptane and octane than on higher paraffinic hydrocarbons

According to Tausz and Donath (151), *Bacterium aliphaticum liquefaciens* utilized all aliphatic hydrocarbons ranging from pentane to decane, but this culture attacked no hydrocarbon lower than pentane Their "Methane bacterium" oxidized not only methane but also hydrogen, ethane, propane, butane, pentane, hexane, heptane and higher hydrocarbons in the aliphatic series with increasing ease It was concluded that when a given member of the series is attacked by a given organism, it may be assumed that all higher members of the series will also be attacked by the same organism The "Paraffin bacterium" described by Tausz and Peter (152) attacked none of the paraffin series lower than hexadecane, $C_{16}H_{34}$, but it readily oxidized triacontane, $C_{30}H_{62}$, tetratriacontane, $C_{34}H_{70}$, and paraffin oil However, the methane oxidizer studied by Münz (*94*) utilized neither ethane nor ethylene

From theoretical consideration based upon thermodynamic reactions, Tausson and Aleshina (*146*) postulated that sulfate-reducing bacteria could utilize no aliphatic hydrocarbon of chain length shorter than decane, $C_{10}H_{22}$ Supporting this view is the experimental work of Novelli and ZoBell (99), who found that decane was feebly attacked by *Desulfovibrio* species which failed to attack lower

hydrocarbons but utilized with increasing ease tetradecane, $C_{14}H_{30}$, eicosane, $C_{20}H_{42}$, docosane, $C_{22}H_{46}$, hentriacontane, $C_{31}H_{64}$, paraffin oil and paraffin wax

TABLE 1

List of microörganisms reported in chronological order by various authors to assimilate paraffin wax

AUTHOR	MICROÖRGANISMS
Miyoshi, 1895 (93)	*Botrytis cinerea*
Rahn, 1906 (104)	*Penicillium* species and other soil fungi
Söhngen, 1913a (121)	*Bacterium fluorescens liquefaciens, B pyocyaneum, B stutzeri, B lipolyticum, B punctatum, Micrococcus paraffinae, Mycobacterium phlei, M album, M luteum, M rubrum, M lacticola, M hyalinum*
Greig-Smith, 1914 (53)	*Bacterium prodigiosus*, soil microflora
Gainey, 1917 (42)	Soil microflora, especially mold fungi
Tausz, 1919 (150)	Microörganisms in soil and canal water
Tausson, 1925a (138)	Mold resembling *Aspergillus flavus* found in soil
Büttner, 1926 (20)	3 species of *Mycobacterium* (probably *phlei, lacticola* and *rubrum*), *Actinomyces chromogenes albus, A bovis, A eppinger, A trautwein*, one mold fungus
Haag, 1926, 1927 (56, 57)	*Mycobacterium* and *Actinomyces* species
Fleming, 1927 (38)	Soil molds and bacteria
Tausson, 1928a (141)	*Penicillium, Aspergillus* and *Pseudomonas* species
Tausson, 1928b (142)	Soil microflora
Jensen, 1931 (75)	*Actinomyces albus, Proactinomyces paraffinae, P agrestis, P polychromogenes, P actinomorphus, P minimus*
Jensen, 1932 (76)	*Proactinomyces corallinus, P salmonicolor, P opacus, P erythropolis, P paraffinae*
Hopkins and Chibnall, 1932 (70)	*Aspergillus versicolor, A flavus, A effusus, A tumari, A parasiticus, A oryzae*
Tausson and Aleshina, 1932 (146)	Sulfate-reducing bacteria from soil
Tausson and Tausson, 1933 (148)	*Aspergillus flavus* and *Penicillum* species
Jensen, 1934 (77)	*Mycobacterium* species
Wackenhut, 1936 (165)	Unidentified bacterium from crude oil
Sturm and Orlova, 1937 (135)	Microörganisms from Ala-Kule Lake
Umbreit, 1939 (100)	*Actinomyces asteroides, A farcinica, A gypsoides* and several *Proactinomyces* species
Tausson, 1939 (137)	*Debaromyces, Hansenula, Endomyces, Torulopsis* and *Monilia* species
Frikson, 1941 (37)	10 strains of *Micromonospora* from lakes
Bushnell and Haas, 1941 (19)	*Mycobacterium phlei, M leprae, M smegmatis, Corynebacterium simplex, C fimi, C tumescens, Penicillium* species and *Pseudomonas* species
Rogers, 1943 (108)	*Micrococcus paraffinae* and microflora of water
ZoBell, *et al* 1943 (181)	Microflora from marine sediments and sea water
Novelli and ZoBell, 1944 (99)	*Desulfovibrio* species from soil and marine sediments

Strawinski and Stone (133) found that hydrocarbons in the range of $C_{10}H_{22}$ to $C_{16}H_{34}$ were oxidized by soil bacteria more readily than those of smaller molecular

weight This observation was confirmed by Stone *et al* (129), who reported further, however, that heavy viscous oils are not utilized as readily as lighter oils This was attributed partly to the fact that the more viscous oils are harder to disperse in liquid media and hence there is less surface exposed to microbial enzymes, but it was believed to be due partly to the difficulty with which the larger molecules in heavy oils are assimilated

The inability of *Aspergillus versicolor* to assimilate larger molecules of paraffin than $C_{34}H_{70}$ was demonstrated by the experiments of Hopkins and Chibnall (70), who obtained results as follows in mineral media enriched with various paraffins finely divided with a microtome

HYDROCARBON	FORMULA	GROWTH
n Tricosane	$C_{23}H_{48}$	Good
n Heptacosane	$C_{27}H_{56}$	Fair
n Nonacosane	$C_{29}H_{60}$	Fair
n Triacontane	$C_{30}H_{62}$	Fair
n-Tetratriacontane	$C_{34}H_{70}$	Slight
n Pentatriacontane	$C_{35}H_{72}$	None
Paraffin wax		Good

Paraffin wax having a melting point of 45° was found to provide for the growth of soil fungi and bacteria better than paraffin wax melting at 56° (104)

Strawinski (132) concluded that the higher the molecular weight, the longer the chain, and the more saturated the compound, the more susceptible aliphatic hydrocarbons up to C_{16} are to dissimilation by mixed cultures of soil bacteria This conclusion was based upon plate counts on mineral media enriched with various hydrocarbons The results of one of his (132) experiments follows

HYDROCARBON	FORMULA	PLATE COUNT PER ML
Cetane	$C_{16}H_{34}$	1,890,000,000
n Pentene	$CH_3{=}CHCH\ CH_2CH_3$	181,000,000
Octene	$CH_2CH{=}CH(CH\ )_4CH_3$	30 000 000
Diisobutylene	$(CH_3)_3CCH_2C(CH_3){=}CH_2$	13 000,000
Trimethylethylene	$(CH_3)_2C{=}CHCH_3$	5,100,000
Iso octane	$(CH_3)_2CH(CH_2)_4CH_3$	5,000,000
Pentene 2	$CH_3CH{=}CHCH_2CH_3$	700 000
n Octane	$CH_3(CH\ )_6CH_3$	10,000

Pseudomonas species and other cultures with which Bushnell and Haas (19) worked grew better on paraffin wax and mineral oils than on kerosene, and better on kerosene than on gasoline or petroleum ether *Mycobacterium* species studied by Haas *et al* (60) grew well on paraffin wax but not on gasoline and only poorly on kerosene Conversely, a *Pseudomonas* species was observed which would grow on petroleum ether, gasoline and kerosene but not as readily on mineral oils or paraffin wax (60)

Using oxygen consumption as a criterion of activity, ZoBell *et al* (181) found

petrolatum and lubricating oils to be oxidized more rapidly by mixed cultures of marine bacteria than kerosene, and kerosene was oxidized more rapidly than gasoline This is in agreement with the observations of Johnson *et al* (79) that *Bacterium aliphaticum* and *Pseudomonas fluorescens* grew with increasing ease on gasoline, kerosene, lubricating oil and paraffin oil *Pseudomonas fluorescens* grew on no hydrocarbon of shorter chain length than dodecane, $C_{12}H_{26}$, but *Bacterium aliphaticum* grew less profusely on dodecane than it did on hexane, heptane, octane or nonane Johnson *et al* (79) speculated that hydrocarbons which are powerful fat solvents may be less readily tolerated or assimilated than those which are less likely to dissolve cell lipids As a case in point, it was related that neither benzene, toluene nor xylene was assimilated by *P fluorescens* or *B aliphaticum*

Whether the common observation that aliphatic hydrocarbons are attacked more readily and by more organisms than aromatic hydrocarbons is attributable to the physical properties or the chemical configuration of the compounds is indeterminate, but a good many microōrganisms are known to attack benzene and its derivatives Stōrmer's (131) *Bacillus hexacarbovorum* utilized toluene and xylene, these benzene derivatives being injurious only when added to mineral media in concentrations exceeding 1 10,000 Wagner's (164) two organisms, *Bacterium benzoli a* and *b*, utilized benzene, toluene and xylene

The ability of soil bacteria to assimilate benzene, toluene, naphthalene and related aromatic compounds generally regarded as antiseptics has been established (52, 73, 91, 116, 136) Wackenhut (165) isolated an organism from Russian crude oil which developed slowly on benzene

Among the numerous hydrocarbon-oxidizing bacteria inhabiting soil in the Baku oil fields of Russia, Tausson (140) found *Bacterium naphthalinicus*, *Bacillus naphthalinicus liquefaciens* and *Bacillus naphthalinicus non-liquefaciens*, which oxidized naphthalene From oil soaked soil Tausson (143) also isolated *Bacillus phenanthrenicus bakiensis* and *Bacillus phenanthrenicus guricus*, both of which utilized phenanthrene besides other hydrocarbons In his review of the literature on the subject Tausson (144) named additionally *Bacillus benzoli* and *Bacillus toluolicum*, which oxidized toluene, xylene and benzene Sulfate reducers have been found to attack slowly naphthalene, phenanthrene and retene (149) The sulfate reducers studied by Novelli and ZoBell (99) were unable to attack naphthalene, anthracene, benzene, xylene or cyclohexane

Most of the *Micromonospora* species studied by Erikson (37) oxidized toluene and naphthalene The oxidation of naphthalene, anthracene, xylene, toluene and benzene by mixed cultures of marine aerobes has been reported (181)

Enrichment cultures of soil bacteria have been found to attack naphthalene, diphenyl, methylnaphthalene, tetralin, butylbenzene and decalin (133) Cetane or *n*-hexadecane was the most rapidly attacked of any of the 18 compounds studied Naphthalene and diphenyl supported bacterial populations only slightly smaller than did cetane

Neither benzene nor xylene were attacked by *Actinomyces oligocarbophilus* (86), which assimilated higher aliphatic hydrocarbons but not the lower ones None

of the pure cultures of hydrocarbon oxidizers studied by Tausz and Peter (152) was able to utilize benzene, toluene or xylene After noting that their *Bacterium aliphaticum* quantitatively decomposed aliphatic hydrocarbons ranging from hexane to tetratriacontane but attacked neither aromatic nor naphthenic hydrocarbons, Tausz and Peter proposed the use of this and related organisms for freeing aromatic and naphthenic hydrocarbons from aliphatic compounds When grown in mixtures of hydrocarbons, *Bacterium aliphaticum liquefaciens* quantitatively utilized aliphatic and naphthenic hydrocarbons, leaving aromatic compounds unaltered This latter organism grew profusely on cyclohexane, methylcyclohexane, 1,3-dimethylcyclohexane and 1,3,4-trimethylcyclohexane

Both *Bacterium aliphaticum* and *B aliphaticum liquefaciens* grew better on caprylene or octene, C_8H_{16}, than on octane, C_8H_{18} (152) This suggests that unsaturated compounds may be more susceptible to microbial attack than saturated ones Haag (56) concluded that unsaturated bonds in molecules are attacked preferentially, after noting that the higher the iodine number of paraffin waxes the more readily they were utilized by *Mycobacterium* species as shown by CO_2 production

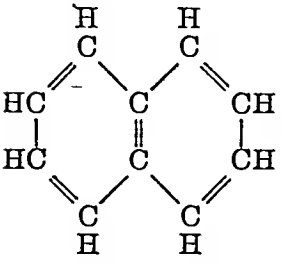
PARAFFIN NUMBER	MELTING POINT	IODINE NUMBER	CO_2 PRODUCED IN 21 DAYS
			mg
1	36°	4 6	309 0
2	40–42°	4 5	290 8
3	44°	4 5	280 6
4	44–46°	4 1	263 3
5	50–52°	3 8	214 4
6	56–58°	2 8	125 1
7	58°	2 8	117 0
8	59°	2 8	122 8
9	60–62°	2 4	96 5
Ceresin	62°	1 8	252 5
Ozocerite	67–69°	1 2	161 3

Ceresin and ozocerite are complex mixtures of hydrocarbons and other compounds Treating paraffins to remove double bonds rendered the resulting compounds less susceptible to attack by bacteria The *Mycobacterium* species studied by Haag developed slightly on amylene or pentene, C_5H_{10}, but not at all on pentane, C_5H_{12}, methane, ethane or ethylene (56)

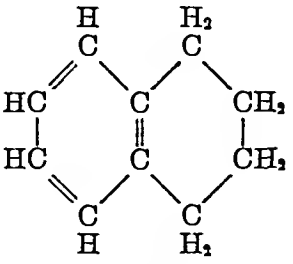
In his studies on the disappearance of ethylene, $H_2C=CH_2$, from ripe apples and bananas, Nelson (96) overlooked the possibility of the microbial consumption of ethylene The susceptibility to microbial attack of natural and synthetic rubbers, which are essentially polymers of unsaturated hydrocarbons, indicates that at least certain kinds of unsaturated hydrocarbons are easily oxidized by microörganisms (179) In recent experiments, we (178) have observed that cetene, $C_{16}H_{32}$, was oxidized from 13 to 30% faster by *Desulfovibrio* species than was cetane, $C_{16}H_{34}$

After noting that naphthalene provided for a better growth of soil bacteria than tetralin and that tetralin was utilized more readily than decalin, Strawinski

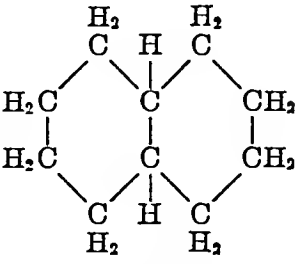
(132) concluded that the saturation of naphthalene reduced the availability of the compound to microbial action



Naphthalene

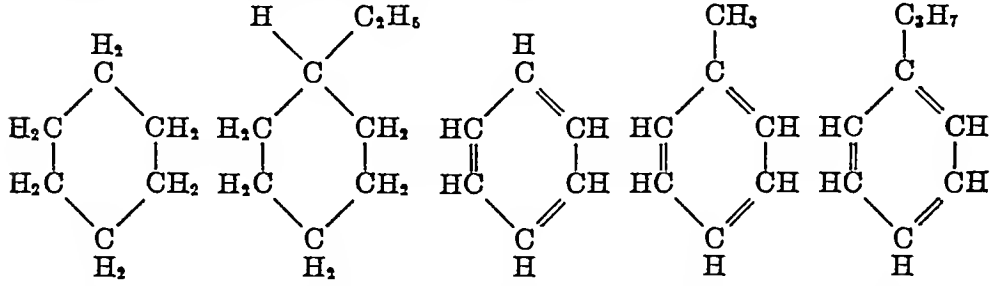


Tetralin



Decalin

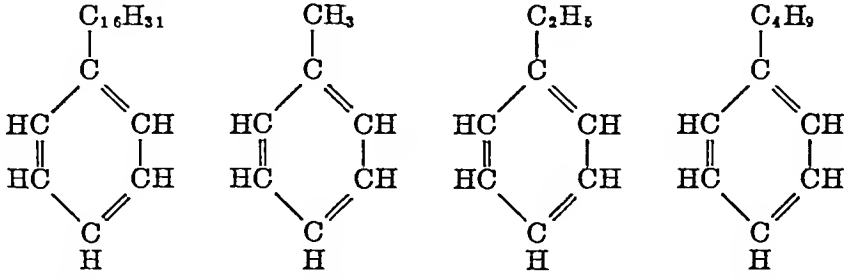
Conversely, both cyclohexane and ethylcyclohexane were utilized more readily than either benzene, toluene or propylbenzene



Cyclohexane Ethylcyclohexane Benzene Toluene Propylbenzene

The bacteria grew better on cyclohexane than on ethylcyclohexane, but growth was much better on either propylbenzene or butylbenzene than on benzene

The fragmentary data on the effect of side-chains or branching on the microbial oxidizability of hydrocarbons are contradictory Strawinski and Stone (133) and Johnson *et al* (79) have presented evidence which indicates that certain organisms utilize *n*-octane more readily than iso-octane (2,2,4-trimethylpentane), but the reverse has been reported for organisms studied by ZoBell (173, 176) Tausz and Peter (152) found that dimethylcyclohexane and trimethylcyclohexane were utilized somewhat more rapidly by *Bacterium aliphaticum liquefaciens* than was cyclohexane On the other hand Tausz and Donath (151) reported that this organism attacked cetylbenzene but not benzene, toluene, ethylbenzene or butylbenzene



Cetylbenzene Toluene Ethylbenzene Butylbenzene

Three of the four strains of *Bacillus toluolicum* studied by Tausson (143) utilized benzene more readily than toluene or ethylbenzene

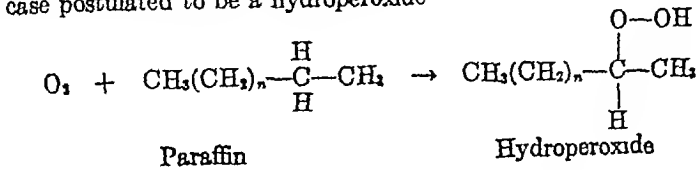
It has been noted that soil bacteria grew fairly well on tertiary butylbenzene, *n*-butylbenzene and isobutylbenzene but not at all on benzene or toluene (133) Either toluene, xylene or phenol was oxidized much more readily by Wagner's (164) *Bacterium benzoli* than was benzene Under comparable experimental conditions 10 g of toluene were destroyed by this organism in 8 days while only 7 g of benzene were destroyed in 38 days The addition of a methyl group to naphthalene has been reported (132) to decrease the availability of the compound to microbial action

According to Matthews (91), the introduction of the methyl group into the benzene ring renders the resulting compound more susceptible to bacterial oxidation She arranged the compounds as follows upon a basis of their tendency to increase the bacterial population of soil, benzene causing the least rise and pinene the greatest

POSITION IN RISING SERIES	NAME OF COMPOUND	EMPIRICAL FORMULA	HEAT OF COMBUSTION
			Kg cal/mol
1	Benzene	C_6H_6	800
2	Toluene	$C_6H_5\,CH_3$	935
3	Xylene	$C_6H_4(CH_3)_2$	1,085
4	Pseudocumene	$C_6H_3(CH_3)_3$	1,241
5	Mesitylene	$C_6H_3(CH_3)_3$	1,252
6	Naphthalene	$C_{10}H_8$	1,235
7	Cymene	$C_{10}H_{14}$	1,414
8	Pinene	$C_{10}H_{16}$	1,489

In interpreting these results Matthews proposed the following explanation, which may be subject to question until more experimental data are available, that "In the assimilation of aromatic compounds the most difficult step is probably breaking of the ring It has been found by experiments that cyclohexane is much less readily attacked than hexane Now for each ring broken benzene gives up 800 units of energy, pinene 1489 Pinene is therefore expected to provide energy more easily than benzene"

The oxidation of a paraffin, under conditions comparable to those encountered by lubricating oils in service in modern engines at 100 to 200°, tends to initiate at a beta carbon atom, that of an alkylnaphthalene at a carbon in the ring to which an alkyl group is attached, and that of an alkyl aromatic at a carbon in an alkyl group adjacent to the ring (182) The initial oxidation product is in every case postulated to be a hydroperoxide



Paraffin Hydroperoxide

Aldehydes, ketones and organic acids are among the principal products formed from the hydroperoxides Mixtures of hydrocarbons do not in general oxidize as might be expected, i e , with the least stable component reacting to the greatest extent On the contrary, the naphthalene derivatives, which by themselves are the most stable, seem to be oxidized preferentially in mixtures Paraffins and naphthenes oxidize at comparable rates when chemically catalyzed at temperatures ranging from 30 to 100° (182) The introduction of a benzene ring at the end of a paraffin molecule causes an increase in rate, as does the introduction of olefinic unsaturation Oxidation rates may be markedly increased by the addition of compounds of copper, lead and iron Similar studies on the factors which influence the oxidizability of various hydrocarbons, the mechanism of the reactions and the end products resulting from microbially catalyzed oxidations are indicated

OCCURRENCE OF HYDROCARBON OXIDIZERS IN NATURE

One of the best sources of hydrocarbon-oxidizing microorganisms is oil-soaked soil or water taken from the bottom of storage tanks containing crude oil or petroleum products Such microorganisms also flourish in the water of petroleum separation tanks and sedimentation ponds Nearly all of the million or so bacteria per ml of water taken from sedimentation ponds in the East Texas field were able to utilize the crude oil emulsified in the brine (178) Equally large populations of hydrocarbon-oxidizing aerobes were found in production waters from separation tanks in the Bradford, Pa , region In a brine sample taken from a sedimentation pond at El Segundo, Calif , an average of 86,000 hydrocarbon oxidizers per ml were found (178)

Bacterial populations as follows were found by Haas *et al* (60) in various source materials

SOURCE MATERIAL	BACTERIA PER ML
Crude oil sediment pond water (Texas)	168 000
Crude oil sediment pond sediment (Texas)	740,000
Waste oil pond (Texas)	480,000
Crude oil soaked soil (Texas)	3 800 000
Tank battery oil soaked soil (Texas)	49,000 000
Kerosene storage tank water (Louisiana)	310 000
Gas oil storage tank water (Louisiana)	1,200 000
Distillate storage tank water (Kansas)	981,000

Other sources from which Haas (58) isolated hydrocarbon oxidizers include water from storage tanks containing gasoline, water from separator pits containing waste petroleum products, sludge from sedimentation ponds, crude oil from pipe lines, crude oil direct from Texas oil wells, water from underground gasoline tanks, water from Supply and Sulfur Springs in Yellowstone National Park, fresh-water wells and ordinary garden soil Approximately 66% of the hydrocarbon-oxidizing cultures were *Pseudomonas* species Among those identified were several strains of *P aeruginosa*, *P borcopolis*, *P fluorescens* and *P striata* Next in order of abundance were species of *Mycobacterium*, *Proactinomyces*, *Actinomyces*, yeast-like organisms and molds (58)

Approximately 95% of the stock cultures of *Pseudomonas* obtained by Haas (58) from various laboratories and the American Type Culture Collection grew in kerosene media, indicating that the ability to use hydrocarbons is a rather general characteristic of this genus *Mycobacterium leprae*, *M phlei* and *M smegmatis* utilized both light and heavy oils and paraffin wax Neither the human nor bovine varieties of *M tuberculosis* developed in hydrocarbon media, but the avian species grew slowly on paraffin

After noting that most *Mycobacterium* species utilize paraffin, while *Corynebacterium* species were unable to do so, Haag (57) proposed paraffin utilization as a characteristic for differentiating these organisms However, several strains of *Mycobacterium* examined by Jensen (75, 76, 77) seemed unable to attack paraffin, although most *Mycobacterium* species did attack paraffin Both Haag (56) and Jensen (77) reported the inability of *Corynebacterium* species to utilize paraffin, but Haas (58) found that *C simplex* and four other unidentified species of *Corynebacterium* grew in media containing either mineral oil or paraffin as the only source of energy

Seventeen strains of acid-fast organisms including *Actinomyces asteroides*, *A farcinica*, *A gypsoides* and 14 strains classified by Umbreit (160) as *Proactinomyces* grew in Czapek's mineral medium enriched with melted paraffin None of the closely related non-acid-fast cultures grew in paraffin media

Lipman and Greenberg (88) reported finding a cocco-bacillus which decomposed petroleum in reservoir fluids coming from an oil well 8,700 feet deep Sulfate reducers, many of which are known to attack hydrocarbons, have been demonstrated in crude oil and oil-well brines (9, 10, 41, 45, 46, 47, 89, 90, 178) From Russian crude oil, a microörganism was isolated which utilized kerosene, benzene, paraffin wax and paraffin oil (165)

The literature summarized in table 1 is indicative of the widespread and general occurrence in soil of organisms which utilize paraffin Söhngen (121) found from 50,000 to 200,000 paraffin-oxidizing bacteria per gram of garden soil and up to 3,000 per ml of ditch water A marked increase in the abundance of microorganisms in soil treated with paraffin was observed by Jensen (75), who noted that nearly all species of *Proactinomyces* found in soil could utilize paraffin, but out of 20 *Actinomyces* tested only *A albus* and *Actinomyces* species 218W and 6S were able to grow on paraffin as a sole source of energy

Species of *Mycobacterium*, *Actinomyces*, *Penicillium*, *Aspergillus*, *Bacillus* and *Bacterium* which attack paraffin were found in nearly all samples of soil, hay, leaves, manure and peat examined by Büttner (20) Microörganisms which attack aromatic hydrocarbons appear to abound in soil as adjudged from the increased microbial populations resulting from the addition of benzene, toluene, naphthalene, etc (52, 73, 91, 116, 136)

By applying crude oil to soil, Baldwin (4) induced the growth of hydrocarbon oxidizers, one of which resembled *Mycobacterium hyalinum* *Bacterium aliphaticum*, originally isolated form European soil (152), was found in New Jersey soil around a gasoline pump (79) Other hydrocarbon oxidizers have been isolated from oil-soaked soil (11, 140, 141, 145)

The widespread occurrence of methane oxidizers in soil is attested by many observations (1, 64, 65, 94, 95, 119, 120, 131, 151, 178) Large numbers of meth-

ane oxidizers have been found in sewage, manure and river mud (43, 44) Kusnetzow (85) credits methane oxidizers in lake water with playing an important rôle in depleting dissolved oxygen

All samples of mud which Erikson (37) collected from Wisconsin lakes contained species of *Micromonospora* which utilized paraffin wax, paraffin oil and various aromatic hydrocarbons Paraffin-decomposing bacteria have been found in Ala-Kule Lake, Russia (135) Bottom deposits from the Dead Sea were found (34) to contain bacteria which utilized crude oil and kerosene In medicinal mud from Tambookansk and Petrosk, Russia, Goobin (49) found, among other hydrocarbon oxidizers, *Bacterium hidium* which attacked ethane, petroleum ether and kerosene

All 0 1 g samples of recent marine sediments and all 60 ml samples of sea water examined by Grant and ZoBell (50) and Novelli (98) contained microörganisms which grew on paraffin oil Species of *Proactinomyces*, *Actinomyces*, *Pseudomonas*, *Micromonospora* and *Mycobacterium* which assimilated hydrocarbons were found (181) to be widely distributed in sea water and marine bottom deposits Microorganisms which can attack rubber hydrocarbons are widely distributed in the sea and in garden soil (179, 180) The hydrocarbon-oxidizing *Desulfovibrio* species studied by Novelli and ZoBell (99) were isolated from marine sediments and garden soil

After noting the general presence of hydrocarbon-oxidizing bacteria in fresh water from various sources, Isjurova (71) cautioned that neither petrolatum nor paraffin oil could be used as sealing agents to exclude oxygen in biochemical oxygen demand tests because these substances are subject to microbial oxidation Furthermore, paraffin oil to a depth of 0 5 to 1 5 cm was found to have little effect on the diffusion of oxygen into underlying fluids

MICROBIAL MODIFICATION OF PETROLEUM

There is ample evidence from field and laboratory observations that crude oil is attacked by soil microörganisms The rapid disappearance of oil from waterways, from soil around refineries, leaking pipe-lines or oil wells, and from polluted beaches is believed to be due largely to the activity of hydrocarbon-oxidizing microörganisms This has been described (173) as Nature's way of "pulling the chain" for the disposal of oil which otherwise would pollute fields and waterways In controlled laboratory experiments the gradual disappearance of oil added to normal soil and marine sediments has been observed (178), while the oil persists almost indefinitely in sterilized soils or sediments

The oxidation of American and Russian crude oils by soil bacteria was observed by Söhngen (121) Under favorable conditions as much as 7 5 mg of oil per square decimeter of oil surface exposed to the culture was oxidized per day at 28° Wagner (164) noted the destruction of 1 g of crude oil in 8 days by *Bacterium benzoli* growing in 100 ml of mineral solution overlayed with crude oil

In 2-liter flasks half-filled with mineral salts solution inoculated with mixed cultures of soil bacteria, Tausz and Peter (152) noted changes in layers of crude oil 1 to 2 mm thick after two or three days' incubation at 25° Within 7 to 14 days the oil layer became a perforated network with holes the size of a pin head.

The threads of the network continued to become finer until they gradually disappeared Part of the oil, pitch-like in color and consistency, sank to the bottom of the flask Layers of crude oil appreciably thicker than 2 mm were not perforated, presumably because bacterial action was retarded by a lack of oxygen Even thin layers of oil remained intact in sterile controls

In an experiment with Mendoza crude oil which originally contained 0 3% of an asphaltic residue insoluble in petroleum ether, Tausz (150) found 5 2% of asphaltic residue after the oil had been acted on by bacteria The view was expressed (150) that besides modifying petroleum in various ways, bacteria contribute to the sinking of oils to the bottom of the ocean Sinking of oil droplets was caused by the increased density resulting from the presence of so many bacteria having a density greater than that of water

After observing the rapid disappearance of crude oil from the waters and beaches of San Francisco Bay following the wrecking of a tanker, Beckman (11) demonstrated the presence of hydrocarbon-oxidizing microörganisms along the beaches in oil-soaked soil, slime and sewage Bacteria and molds caused significant changes in the specific gravity and viscosity of oils in a month at 40° Crude oil emulsions were broken by proteolytic bacteria, suggesting (11) that in these cases the emulsifying agent was of protein nature Allegedly the changes occurred under anaerobic as well as aerobic conditions

The slow destruction of petroleum by several species of soil *Actinomyces*, three species of *Mycobacterium* and a mold was observed by Büttner (20) Tausson (141) expressed the belief that crude oils in nature undergo progressive oxidative modification and destruction until microbial activity is arrested by conditions in oil pools *His belief was based upon the general abundance of hydrocarbon* oxidizers in oil-soaked soil and upon observed changes in samples of petroleum acted on by microörganisms Thermodynamic considerations and experimental results with sulfate reducers suggested to Tausson and Aleshina (146) that, under anaerobic conditions, sulfate reducers tend to convert paraffinic hydrocarbons containing 10 or more carbon atoms per molecule into naphthenic compounds Conclusive proof is still lacking Rogers (107) expressed the belief that paraffinic hydrocarbons are oxidized and polymerized to yield naphthene and asphalt base compounds and that sulfate in oil-field water is reduced to sulfide, reactions which might be catalyzed by sulfate reducers

Microörganisms oxidized Emba crude oil at an average rate of 250 g per square meter of surface exposed to the culture in seven months (147) It is pointed out that, since the oils under investigation consisted mainly of naphthene and polynaphthene hydrocarbons, the disappearance of 45% of the amount initially present established that microörganisms could utilize such hydrocarbons Microbial activity was found to cause changes in the following properties of Emba crude and cylinder oil prepared therefrom index of refraction, iodine number, saponification number, density and physical appearance *The middle fractions* were oxidized more rapidly than the heavy ones, resulting in an accumulation of the latter (147) In the beginning of the development of bacteria in oil, an intensive consumption of unsaturated compounds took place as indicated by the

decrease in the iodine number With further development, the iodine number increased slowly, indicating a relative accumulation of unsaturated hydrocarbons and speaking for the view of Tausz (150) on the formation of unsaturated hydrocarbons as intermediate products in the oxidation of saturated hydrocarbons A sharp and great increase in the saponification number with the development of bacteria pointed to the formation of higher fatty acids and naphthenic acids, and this was regarded (147) as the cause of the formation of water emulsions of oil products invariably observed in such cultures

The utilization and modification of 31° Iles Dome crude oil by both aerobic and anaerobic bacteria has been reported (92) Ordovician oil-well water, Dakota oil-well water and water in which Frontier shale had been leached for several days was enriched with crude oil and inoculated with mixed cultures of soil, lake-slime or ditch-water microörganisms Every combination except the sterile controls resulted in visible changes in the oil The opinion was expressed (92) that over a considerable period of time, drops of oil of super-capillary size can be oxidized by microörganisms to CO_2 and water under the conditions which have prevailed in the reservoir rocks

Pronounced changes in a variety of Russian crude oils as a result of the activity of the sulfate reducer, *Desulfovibrio aestuari* have been observed (89) Sulfur compounds were preferentially attacked and the crude oil itself was slowly oxidized

The widespread distribution and great diversity of hydrocarbon-oxidizing microörganisms in soil, water and recent sediments and their demonstrated ability to function throughout a wide range of environmental conditions quite definitely establish that, in the absence of inhibiting agents, petroleum hydrocarbons can be expected to be modified or completely destroyed Indications are that these changes take place far more rapidly and extensively in aerobic than in anaerobic environments The activities of hydrocarbon-oxidizing microörganisms may help to explain why Trask (158) and others have failed to find petroleum hydrocarbons in recent sediments

Conditions inimical to the activity of microörganisms appear to be prerequisite to the accumulation of petroleum hydrocarbons in recent sediments It has been found (178) that a good many samples of petroleum and more samples of oil-well brines have bacteriostatic properties, the exact cause of which is still unknown Experiments now in progress at the Scripps Institution (178) indicate that certain heavy metals, the presence of H_2S, low redox potentials (175) and specific oxidase inhibitors prevent the microbial oxidation of hydrocarbons This suggests that in the search for source sediments of petroleum and the origin of oil, particular attention should be given to substances or conditions in sediments which are inimical to the activities of hydrocarbon-oxidizing microorganisms As academic examples of inhibitory substances may be mentioned cyanide and urethane, low concentrations of which were found (79) to inhibit the oxidation of hydrocarbons by *Bacterium aliphaticum*

Little is known regarding the occurrence or activity of bacteria in petroleum deposits Sulfate-reducing bacteria, some of which attack hydrocarbons, have

been found in oil-well brines from various depths (9, 10, 45, 46, 47, 89, 90, 178) In petroleum from a well 8700 feet deep, Lipman and Greenberg (88) found bacteria which completely decomposed samples of petroleum with the formation of CO_2 Sulfur bacteria have been found in oil from a depth of 6000 feet (72) It is a moot question, however, whether bacteria in reservoir fluids coming from oil wells are species indigenous to subterranean reservoirs or if they are adventitious species introduced during drilling or production operations The continued abundance of bacteria in reservoir fluids for prolonged periods after anything has been introduced into the well, the peculiar types of organisms found, evidence of activity such as a depletion of sulfates (118) and demonstrated ability of the bacteria to function under similar environmental conditions all speak in favor of the occurrence and activity of bacteria in petroleum reservoirs

Living bacteria are most abundant in Tertiary formations and gradually disappear in older Cretaceous, Jurassic, Permo-Triassic and Permian formations according to Ginsburg-Karagitscheva (46) This worker, like Rogers (106), interpreted the absence of sulfates from certain oil-well waters as evidence of earlier bacterial activity

The demonstrated ability of anaerobic sulfate reducers to attack hydrocarbons (99, 146, 149, 178) lends considerable weight to the argument that bacteria may modify petroleum after its formation The activities of hydrocarbon-oxidizing microörganisms could account for many observed and anomalous conditions For example, the preferential microbial attack of paraffinic hydrocarbons in the middle range could explain the occurrence of deposits of tars associated with gas typified by extensive deposits in Venezuela and Mesopotamia The preferential attack of unsaturated hydrocarbons could account for the general absence of olefines in crude oil Environmental conditions conducive to the activity of bacteria which completely destroy oil could account for certain areas being barren of oil fields which possess all the known geological characteristics of areas containing prolific oil fields The reasonableness of this latter possibility is vouchsafed by geological conditions which led Thom (156) to conclude that it is much more reasonable to regard the metamorphic destruction of oil pools as having occurred progressively and gradually rather than suddenly

In summarizing the rôle of bacteria in the formation of petroleum, ZoBell (173, 177) concluded that the release of oil from sediments is one of the most important functions of bacteria in the accumulation of oil in subterranean deposits Bacteria release oil from sediments and promote its migration and accumulation by dissolving carbonates, producing CO_2 and detergents and by the physical displacement of oil from solids by thigmotaxis The accumulation of oil tends to preserve it from microbial attack because the microörganisms and their enzymes are active only in the presence of water Baier (3) envisioned sulfate-reducing bacteria growing at the oil-water interface in petroleum deposits where paraffinic constituents might be converted into oxybitumens or asphaltenes and gas Although certain constituents of petroleum were shown to be germicidal, Baier (3) concluded that at some little distance from the oil-water interface the concentration of toxic constituents would be low enough to permit the

activity of hydrocarbon-oxidizing microörganisms Sanders (110) expressed the belief that petroleum deposits provide an aseptic environment for the prolonged preservation or embalmment of the organized remains of certain biological materials

It is an anomalous situation that the petroleum industry, praiseworthy for its many outstanding scientific and technological achievements, has devoted so little attention to the effects of microörganisms on petroleum or its products Intensive and extensive microbiological studies are needed to elucidate the theories of the origin and occurrence of crude oil Besides being of academic interest, information gained from such studies may find important application in the discovery, recovery, refining, modification and exploitation of petroleum or its products Urgently needed is reliable information on the relative susceptibility of various kinds of hydrocarbons to microbial attack and the nature of the resultant end products under different environmental conditions

MODIFICATION OF PETROLEUM PRODUCTS

The microbial oxidation of gasoline, kerosene, lubricating oil or similar refined petroleum products has been quite commonly observed (19, 20, 49, 79, 121, 144, 147, 151, 155, 165, 181) Even heavy residues such as asphalt are susceptible to microbial attack (129, 178) The observations suggest that wherever petroleum products are stored in the presence of water for prolonged periods, the possibility of microbial modification exists

In the water bottoms of kerosene and gasoline storage tanks, Thaysen (154) found nitrate reducers which fermented kerosene with the formation of methane and ethane Besides causing the corrosion of metal tanks, sulfate reducers in the water bottoms contaminated the gasoline with undesirable H_2S A spontaneous explosion in a kerosene storage tank was attributed by Thaysen to the microbial formation of an explosive mixture of gases

Microbial activity may be a factor in gum formation in high test gasolines and the production of "off colors" in water-white distillates (58) Diesel fuels and mineral oil are probably more susceptible to microbial modification than gasoline or kerosene Haas (58) stressed the possible significance of so many workers reporting the production of oil-soluble acids in petroleum or its products as a result of microbial activity

Upon examining the lubricating oil and diesel motor fuel from marine engines which had been out of commission for several months, large numbers of hydrocarbon-oxidizing bacteria were found (178) which could have been responsible for the emulsified and corroded condition of the water-contaminated engine oils Similar circumstantial evidence has been obtained for the deterioration of transformer oils Such oils stored in the presence of water for prolonged periods are often emulsified, presumably through bacterial activity

Mycobacterium No 24 studied by Haas (58) produced 2 14 mg of carotenoid pigments in 500 ml of oil within two weeks, imparting an orange color to the oil A highly chromogenic strain of *Proactinomyces rubropertinctus* was also isolated from water in oil storage tanks (60) Among the hydrocarbon oxidizers studied

by Söhngen (121) red, orange and yellow pigments were produced by *Mycobacterium rubrum*, *M phlei* and *M luteum*, respectively Some of the hydrocarbon-oxidizing species of *Actinomyces* and *Mycobacterium* observed by Haag (56) were pigmented The formation of a brown pigment soluble in alcohol indicated to Rahn (104) that paraffin was being used by *Penicillium* species The growth of *Aspergillus versicolor* (70) on paraffin was accompanied by the production of first yellow and then red pigment An olive-green pigment was produced in cetane cultures, reddish-orange in naphthalene cultures and yellow-brown pigment in tetralin, decalin and diphenyl cultures studied by Strawinski (132) These examples of pigment production by microörganisms growing on petroleum products should suffice to indicate the possibilities of discoloration

Observed decreases in the octane rating of aviation gasoline stored over water were attributed (173), at least in part, to bacteria which either (a) preferentially attack branched-chain hydrocarbons which have the highest anti-knock characteristic, (b) produce sulfides which precipitate lead tetraethyl or (c) produce peroxides which catalyze the deterioration of lead tetraethyl

Though not definitely establishing that the hydrocarbons were oxidized as the source of energy, Kegel (84) found that the bacterial reduction of sulfates in natural gas being cooled by direct contact with water in trickling gas coolers resulted in an undesirable increase in the H_2S content of the gas By the strategic placement of medium inoculated with *Methanomonas methanica*, Yurovskii *et al* (169) claimed that 96% of the methane in coal mines was destroyed under experimental conditions

Much trouble is caused by the growth of bacteria in mineral oil emulsions used as cooling agents in the cutting and grinding of metals in machine shops The emulsions may be broken, sour objectionable odors sometimes develop, and machinists may become afflicted with dermatitis Lee and Chandler (87) found 15,000,000 to 50,000,000 bacteria per ml of cutting compound Predominating was *Pseudomonas oleovorans*, which, like other bacteria isolated from the cutting compound, *readily utilized the emulsified oil* Naphthenic acids, used as emulsifying agents in the cutting compound, were also utilized by the bacteria as a source of food Coal tar disinfectants were ineffective, but the addition of 15 pounds of resorcinol per month to a 500 gallon tank prevented the spoilage of the cutting compound

Duffett *et al* (31) found from a million to 350 million bacteria per ml of cutting oil emulsions, including six new species of *Pseudomonas*, *P oleovorans*, two species of *Achromobacter*, *Bacillus alvei*, yeasts and molds Bacteria growing in mineral oils have been credited as being the causative agents in industrial dermatitis (30) Cutting oils give rise to a great deal of annoyance and increased labor turnover in machine shops because of epidemics of boils and furuncles (128) The cutting oils become heavily contaminated with suppurative organisms, the most serious of which is *Staphylococcus aureus* The paraffin oil used in cutting oils is the one forming the most favorable culture medium for these organisms (128) The rancidity and allergenic properties of ointments and other pharmaceutical preparations containing mineral oil or petrolatum have been attributed to the action of hydrocarbon-oxidizing microörganisms

Hydrocarbon-oxidizing bacteria may be responsible for the fairly rapid disappearance of oils sprayed on various kinds of foliage as a carrier of insecticides Second and third applications of spray oils generally disappear more rapidly than the first

The finding in soil underlying defective spots in old asphalt-paved highways of exceptionally large populations of bacteria, many of which are endowed with the ability to oxidize asphalt in the laboratory, suggests that microbial activity may contribute to the deterioration of such highways There is similar circumstantial evidence to incriminate bacteria as being responsible for the disappearance of asphalt in contact with soil under concrete foundations and other structures under which a layer of asphalt had been applied as a waterproofing agent When mixed with normal soil, asphalt is slowly decomposed (178), a change which does not occur in sterile soil

ACTION ON RUBBER HYDROCARBONS

The tacit assumption that rubber stoppers, tubing, gaskets, etc , are biologically inert has led to anomalous experimental results For example, Hatfield and Morkert (66) observed that rubber stoppers materially increased the biochemical oxygen demand of water samples This observation was confirmed by ZoBell and Grant (180), who noted further that exposure to pure gum rubber, duprene or neoprene increased the oxygen consumption by bacteria in sea water In light of the information summarized in the following paragraphs, it is believed that the distilled water in the experiments of Bigger and Nelson (14) was rendered growth-promoting for certain coliform bacteria because the bacteria in question assimilated rubber hydrocarbons The growth-promoting properties of rubber tubing and stoppers were attributed (14) to the talc dressing, which was believed to adsorb nutrients from the atmosphere

The utilization of caoutchouc by *Mycobacterium* species was reported by Söhngen (121) more than thirty years ago From garden soil and canal water, Söhngen and Fol (123) isolated strains of *Actinomyces fuscus*, *A alba*, *A chromogenes*, *A elastica* and other *Actinomyces* species which grew well on purified rubber treated with mineral solution In such media there was a fair growth of *Mycobacterium rubrum*, *M lacticola*, *Bacillus mesentericus* and *Pseudomonas fluorescens*

Arens (2) attributed the red spots which appeared on unpreserved sheet rubber to the growth of *Serratia marcescens* This organism, along with other chromogenic bacteria, was isolated from damaged crepe rubber (28) Further work (17, 32) established that the "spot disease" of sheet or crepe rubber was due to microörganisms Unlike mildews and molds which grow primarily on the surface of moist crude rubber at the expense of nitrogenous impurities therein, the "spot disease" bacteria decomposed the rubber

In soil and on rubber plants, Novogrudski (100) found actinomyces and bacteria which decomposed caoutchouc One of these organisms was described as an orange-yellow coccus which decomposed caoutchouc readily in mineral solutions at pH 7 to 8 5 but not at pH 6 3 From fermenting hevea latex, Corbet (24) isolated *Gaffkya tetragena*, *Alcaligenes denitrificans*, *Micrococcus eatoni*, *M ridleyi*, *M*

epimetheus, M chersonesia, Bacillus pandora and *Torulae heveae*, each of which oxidized rubber hydrocarbons The gradual loss in weight and decreased elasticity of moist sheets of plantation crepe rubber have been attributed (26, 27) to the activities of *Penicillium* and *Aspergillus* species

Unexpectedly low yields of rubber from guayule latex under certain conditions of storage have been caused by the activities of soil microörganisms, some of which destroyed as much as 15%, and adversely affected all, of the rubber hydrocarbon in six weeks at 37° (125) Spence (124) described four species of *Actinomyces* and several strains of anaerobic bacteria which decomposed the rubber hydrocarbons in guayule latex Spence and van Niel (126) characterized the decomposition of hevea latex hydrocarbons as being both rapid and profound after noting the disappearance of 20% of the rubber in purified latex under the influence of soil microflora for six weeks at 20°

From 20 to 40% of the rubber from latex dispersed in mineral solution was destroyed in a month at 28° by *Actinomyces aurantiacus, A longisporus ruber, Aspergillus oryzae, Penicillium* species and various raw soil cultures (82) Neither *Azotobacter chroococcum, Pseudomonas fluorescens, Bacillus mycoides, Sarcina ureae* nor *Proteus vulgaris* was able to attack rubber hydrocarbons By sprinkling finely divided soil on plates of mineral agar coated with a thin film of rubber, Kalinenko (82) showed that rubber-oxidizing bacteria are widely distributed in garden soil

ZoBell and Beckwith (179) found purified rubber to be oxidized by the following pure cultures *Bacterium aliphaticum, Pseudomonas fluorescens, P neritica, Vibrio marinofulvus, Serratia marcescens* and certain strains of *Escherichia coli* Negative results were obtained with *Proteus vulgaris, Alcaligenes faecalis, Sarcina lutea* and several strains of *E coli* Mixed cultures of soil bacteria attacked highly purified caoutchouc, neoprene, duprene, butaprene, chemigum, amerpol, hycar, thiocol RD, butyl rubber and various experimental elastomers synthesized from butadiene, $CH_2{=}CH{-}CH{=}CH_2$, isoprene, $CH_3{-}CH{=}CH{-}CH{=}CH_2$, isobutylene, $(CH_3)_2C{=}CH_2$, acrylonitrile, $CH_2{=}CHCN$, or styrene, $C_6H_5{-}CH{=}CH_2$ In one experiment 78% of the rubber initially present was oxidized to CO_2 and water and from 10 to 20% of it could be accounted for as bacterial protoplasm

As a general rule, pure rubber is oxidized by bacteria more rapidly than compounded or vulcanized rubber products The rate of oxidation depends to a large extent upon the surface exposed to the culture In order to retard or prevent the microbial deterioration of rubber products, Dimond and Horsfall (29) advocated the use of anti-microbial agents in compounding rubber, particularly rubber which is to be used in an aqueous environment It is not a simple problem because chemicals which are ordinarily effective fungicides may be inactivated by the rubber hydrocarbons, accelerators of vulcanization, antioxidants or fillers used in compounding rubber Tetramethylthiuram disulfide, an accelerator known as Tuads, rendered rubber resistant to attack by *Macrosporium sarcinaeforme*

ACTIVITIES OF HYDROCARBON OXIDIZERS IN SOIL

The deduction, that hydrocarbonoclastic microörganisms are active in most normal soils, is based upon the general abundance of hydrocarbon oxidizers in soil (to which reference has been made on the preceding pages) and the apparent destruction of hydrocarbons known to be produced by plants A great variety of aliphatic hydrocarbons ranging from C_7H_{16} to $C_{35}H_{72}$ are known to occur in plant and insect waxes (23, 117) Extensive search for rubber-producing plants during the War years has shown that most plants contain some hydrocarbons, and certain plants such as guayule, milkweeds and dandelions, for example, contain appreciable quantities of hydrocarbons It has been estimated that an average of 0 02% of the solids produced by plants consist of hydrocarbons There is also evidence that soil bacteria produce several hydrocarbons besides methane (74, 173, 177, 178) Inasmuch as these hydrocarbons ordinarily do not accumulate in the soil, it is inferred that they must be attacked by microörganisms If the hydrocarbons produced by plants since Cambrian times had been preserved, a goodly portion of the earth's carbon would have long ago been tied up In certain environments where conditions have not been conducive to the activities of hydrocarbon oxidizers, petroleum is believed to have accumulated in structural or stratigraphic traps

Methane production (5, 6) commonly occurs in soil, and, under favorable conditions, much methane may be oxidized by bacteria After observing an increase in the organic content of mineral solutions in which methane oxidizers were growing, Giglioli and Masoni (43) suggested that biological methane oxidation in nature continually contributes to the organic fertility of soil The effect of methane oxidizers on the organic content of soil has been studied by Harrison and Aiyer (64) and Aiyer (1)

Two- to threefold increases have been observed (63) in the nitrogen and organic content of soil exposed for several years to natural gas from buried conduits After establishing that the increases were not due to the presence of ammonia or nitrogen oxides in the gas, the increases were attributed to the effect of methane on the growth of microörganisms Increases in the nitrogen and organic content of soil around leaking gas mains, attributable to biological activity, have also been observed (113)

A few days after the addition of paraffin wax to soil, Gainey (42) noted a decrease in the content of nitrate and ammonia The amount of paraffin oxidized was limited primarily by the supply of available nitrogen It is entirely possible that had the experiments been conducted over a period of weeks or months until the dead cells of paraffin oxidizers had undergone decomposition, he would have found, like others (63, 113), that treating soil with hydrocarbons had a beneficial effect on its organic and nitrogenous fertility

The application of crude petroleum to soil was found (4) to increase the total bacterial population, but the number of bacterial types was greatly reduced The multiplication of aerobes was stimulated more than of anaerobes The petroleum appeared to be broken down into simpler products and gradually disap-

peared Up to 50% of the petroleum had disappeared in 56 days from 4000 g of soil treated with 200 ml of crude petroleum having a density of 0 8370 During this period Baldwin (4) observed that both ammonia production and nitrate formation were inhibited In field experiments it was found that the largest and best ears of corn were produced in hills treated with petroleum Similarly, Carr (21) found that applications of crude petroleum to soil improved the growth of soybeans Up to 30 ml of petroleum per gallon of soil, or 0 75% , was beneficial, and petroleum was not injurious until five times this concentration was added to soil The damage to plants resulting from the application of 4% or more of petroleum was explained (21) as being due to the inability of plants to obtain water fast enough to meet their requirements

It has been commonly observed that vegetation is killed by the intentional or accidental application of relatively large quantities of oil on soil The lethal concentration is a function of the kind of oil, soil conditions, method of application, season, kind of vegetation, humidity, and other factors However, it is only a matter of a few weeks until the arability of the soil has been restored, presumably due to the microbial oxidation of the oil, and there are many reports of the fertility of the soil having been improved by the oil The systematic application of sublethal concentrations of oil may prove to be beneficial under certain circumstances For controlling weeds in crops of carrots, parsnips and celery, the application of 65 to 85 gallons of stove oil per acre is required and more than one such application may be necessary during the growing season From 100 to 300 gallons of oil per year has been applied to the soil for the control of weeds in citrus orchards Hildebrand (69) gives several references to the recent literature on the use of petroleum oils for controlling weeds

Truffault and Bezssonoff (159) found a *Bacterium B*, which could utilize either paraffin oil, cyclohexane or methylcyclohexane as a source of energy, growing symbiotically with the anaerobic nitrogen fixer, *Clostridium pasterianum* The hydrocarbon-oxidizing symbiont closely resembled *Bacterium aliphaticum* of Tausz and Peter (152)

After noting the rapid microbial oxidation of paraffin wax in the soil, Gainey (42) emphasized the often overlooked fact that data obtained by methods which call for the use of wire baskets, pots, stakes or other experimental apparatus rendered "inert" by coating with paraffin prior to burial in the soil may be invalidated by the action of microörganisms on paraffin Similarly, Rogers (103) has pointed out that coating experimental apparatus with paraffin is a source of error in corrosion tests in water because of the susceptibility of paraffin to microbial oxidation

Until he learned that the hydrocarbons were themselves rapidly attacked by soil organisms, Greig-Smith (53) was surprised to find that coating dried blood or casein particles with petrolatum or paraffin did not prevent such particles from being attacked by soil bacteria According to Fleming (38), paraffin could not be used as a coating to decrease the injurious action of lead arsenate on plant roots because the paraffin was rapidly decomposed by soil microflora The growth of molds was stimulated by the addition of paraffin to soil much more than was the growth of bacteria

Jensen (75) reported that the addition of paraffin to soil caused a marked increase in the microbial population, the abundance of *Proactinomyces* species being particularly increased The following organisms were described as being able to oxidize paraffin *Proactinomyces actinomorphus, P agrestis, P minimus, P paraffinae* and *Actinomyces albus*

Treating soil with toluene resulted in a temporary decrease followed by a great increase in the bacterial population, according to Russell and Hutchinson (109) Following the application of toluene to soil, the plate count rose from an initial count of 5 to 9 million per gram to 40 million or more Toluene also caused an increase in ammonia production An increase in the content of soluble organic matter in soil was observed by Pickering (103) to result from treating soil with benzene or paraffin oil These workers like Buddin (18), who noted a great increase in the bacterial population and ammonia production in soil treated with M/200 to M/2 concentrations[2] of toluene, benzene, cyclohexane, pentane, hexane, phenol, cresol, quinone, hydroquinone, pyridine, alcohol, ether or acetone, attributed the beneficial effects on soil fertility to the destruction of protozoa or some other inimical factor Evidence was obtained by Sen Gupta (116), however, which indicated that such aromatic antiseptics were oxidized by soil bacteria Phenol and cresol were observed to disappear from normal soil but not from sterilized soil, and the second and third applications of these substances disappeared much faster than the first application

It was finally established by Matthews (91) that while the "antiseptic" hydrocarbons or derivatives promoted the growth of bacteria by destroying predatory protozoa, such substances as toluene, benzene, xylene, pseudocumene, mesitylene, naphthalene, cymene, pinene, hexane, chlorobenzenes, nitrobenzenes and cresols provided energy for the multiplication of certain soil bacteria independent of any effects on protozoa Tenfold or greater increases in the bacterial population followed the application of M/10 concentrations[2] of benzene *Bacillus liquefaciens* could tolerate and attack nearly any of the aforementioned compounds, but most species of soil bacteria exhibited a high degree of specificity in their ability to assimilate various kinds of hydrocarbons Aeration promoted the bacterial oxidation of the compounds

The addition of naphthalene to soil was found by Tattersfield (136) to cause decreases followed by large increases in the number of bacteria Microbial multiplication and the decomposition of naphthalene were promoted by aeration About 50 days were required for the disappearance of 50 mg of naphthalene from 100 g of soil initially treated, but the second 50 mg of naphthalene disappeared in only 20 days and the third 50 mg disappeared in 10 days

Jacobs (73) described experiments in which the aerobic bacterial population of soil increased from an initial count of a few millions per gram to more than three billion per gram in two or three days following the application of M/10 naphthalene to soil Within a week or two the bacterial population dropped to around 400,000,000 per gram Several strains of bacteria were isolated which were capable of using naphthalene as a sole source of energy The naphthalene oxidizers preferred ammonium to nitrate as a source of nitrogen The ammonia

[2] The stated concentrations represent moles of hydrocarbon per kg of soil

content of the soil treated with naphthalene at first decreased and then increased. It was explained (73) that the naphthalene-decomposing bacteria utilize the available nitrogen in the soil, then upon the exhaustion of the naphthalene the bacteria die and undergo decomposition Potential energy added to the soil in the form of naphthalene provided for the activities of nitrogen fixers, thus resulting in an overall increase in the nitrogen content of the soil

The observed increases in the bacterial populations of soils treated with certain aromatic compounds have been attributed by Gray and Thornton (52) to the bacterial utilization of the aromatic compounds Out of 245 soil samples examined, 146 yielded bacteria which could oxidize either naphthalene, phenol or cresol Some of the 208 strains of such bacteria, representing 7 genera and 25 species, could oxidize toluene, phloroglucinol or resorcinol Described as new species which could utilize either naphthalene, phenol or cresol were organisms now listed in the Bergey (12) Manual as *Achromobacter cycloclastes*, *A iophagum*, *Actinomyces convolutus*, *Bacillus closterodes*, *B platychoma*, *Micrococcus piltonensis*, *M sphaeroides*, *Mycoplana bullata*, *M dimorpha*, *Proactinomyces actinomorphus*, *P agrestis*, *P coeliacus*, *P crystallophagus*, *P erythropolis*, *Pseudomonas arvilla*, *P boreopolis*, *P crucivae*, *P dacunhae*, *P desmolyticum*, *P pictorum*, *P rathionis*, *P salopium*, *Vibrio cuneatus*, *V cyclosites* and *V neocystes* *Proactinomyces globerulus* has been described as a phenol oxidizer (51)

MICROÖRGANISMS AS INDICATORS OF OIL DEPOSITS

Anomalies in the appearance of vegetation over and surrounding oil fields are often noticeable, particularly from a scouting plane While unquestionably these anomalies are in many cases due to the underlying rock formation, in some cases differences in vegetation are believed to be due to the effect of hydrocarbon-oxidizing microörganisms upon the organic fertility, nitrogen content, redox potential, water-holding capacity, particle aggregation or other properties of the soil Wherever careful tests have been made, it has been found that hydrocarbons are slowly escaping from subterranean deposits Conspicuous surface seeps of gas, oil, asphalt or tars have provided clues to the discovery of some oil fields, but hydrocarbons have been detected in the soil overlying other oil pools only by the most meticulous analytical procedures The sensitivity of hydrocarbon-oxidizing microörganisms as indicators of traces of hydrocarbons in soil is the basis of so-called "geomicrobiological prospecting" methods

In the geomicrobiological prospecting method of Sanderson (111), patented receptacles, containing mineral media inoculated with bacteria which utilize volatile hydrocarbons, are introduced in the soil in holes dug to a depth at which hydrocarbonogenetic bacteria such as methane producers are not active The growth of bacteria on the media is indicative of the presence of volatile hydrocarbons in the sub-soil Sanderson also proposed analyzing soil for its content of bacteria which oxidize volatile hydrocarbons, believing that the abundance of such bacteria would bear some relationship to the presence of petroleum hydrocarbons escaping from subterranean deposits Blau (16) has patented a method of geomicrobiological prospecting in which certain chemical agents are alleged to

produce characteristic color changes when applied to soil in which hydrocarbon-consuming bacteria have been active He named *Bacillus ethanicus* as an organism which utilizes ethane in soil

Nearly any method of prospecting by chemical assay or "soil analysis" which ignores the effect of bacteria on the hydrocarbon content or other properties of soil can be expected to give anomalous results under certain conditions Hydrocarbons may be decomposed or altered by microörganisms in the soil almost as fast as the hydrocarbons enter the biosphere from subterranean deposits The rapidity with which hydrocarbons are decomposed in soil may vary seasonally with the water content of the soil, its oxygen content, abundance of organic matter, nitrogen content, temperature, growth phase of the organisms and other factors which influence the abundance, kinds and activity of microörganisms in soil The rapidity with which various kinds of gaseous, paraffinic and aromatic hydrocarbons are destroyed in soil samples is attested by results summarized in the foregoing paragraphs

Hydrocarbon-oxidizing microörganisms are believed to have been instrumental in the formation of "paraffin dirt", a kind of waxy soil sometimes found overlying subterranean deposits of petroleum Samples of "paraffin dirt" have been found (178) to contain large numbers of bacteria (predominantly species of *Proactinomyces*, *Mycobacterium* and *Desulfovibrio*) and complex waxes Apparently the decomposition of the material has been arrested by the accumulation of toxic metabolic products of microörganisms or antibiotic substances and protection from contact with water or air by an outer coating of waxes and heavy hydrocarbons The latter are probably carried to the soil surface dissolved in lighter hydrocarbons escaping from subterranean deposits Some of the hydrocarbons may be oxidized by microörganisms, the cell substance of which is added to the mass of the partially preserved material

BACTERIOSTATIC HYDROCARBONS AND DERIVATIVES

When mixtures of colon-typhoid organisms were treated with petroleum ether at room temperature for 15 minutes, Bierast (13) observed that typical strains of *Escherichia coli* were selectively killed while typhoid and paratyphoid organisms were not injured by this treatment Gaertner's bacillus responded virtually the same as typhoid organisms After confirming these observations with a large number of stool specimens from typhoid patients, Hall (61) proposed the use of petroleum ether for demonstrating typhoid organisms in clinical material The organisms or stool specimens were placed in a tube of broth, treated with an excess of petroleum ether and shaken for half an hour After standing for two hours at room temperature cells of *E coli* were almost quantitatively killed while typhoid organisms survived, provided the boiling point of the reagent was less than 50° Petroleum ethers having higher boiling points such as benzine (b p 80°–90°), ligroin (b p 100°–110°), pure heptane (b p 98°) and octane (b p 124°) were not specific in their effects on colon-typhoid organisms Pentane (b p 38°) added to nutrient broth completely inhibited *E coli* and stimulated the multiplication of typhoid organisms Irregular results were obtained with dysentery and para-

dysentery bacilli The cholera vibrio and members of the *Proteus* group were inhibited by petroleum ether of low boiling point in the same manner as *E coli*

Schuscha (115) was successful in detecting typhoid organisms in polluted water when the ratio of typhoid organisms to *E coli* was as high as 1 5000 by precipitating the organisms with iron salts and then treating the precipitate with petroleum ether The latter inhibited *E coli* while permitting the free multiplication of typhoid organisms in nutrient solutions These observations were confirmed by Heyn (68) who found further that twelve hours agitation with petroleum ether rendered *E coli* cultures sterile After such treatment cells of *E coli* as well as diphtheria bacilli were almost quantitatively transferred from the aqueous medium to the petroleum ether

Walbum (166) failed to find a marked difference in the susceptibility of enteric organisms to petroleum ether, although the typhoid bacillus was somewhat more resistant than *E coli* For example, in one experiment 24% of the typhoid organisms survived treatment with petroleum ether as compared with a 12% survival of *E coli* Walbum concluded that the germicidal effect of petroleum fractions is related to their boiling point The following percentage survival of typhoid bacilli was found (166) after exposure in broth cultures for two hours to various pure hydrocarbons

HYDROCARBON	% SURVIVAL	HYDROCARBON	% SURVIVAL	HYDROCARBON	% SURVIVAL
Pentane	13 4	Cyclohexane	2 1	Butylbenzene	50 8
Hexane	32 4	Menthene	0	Cymene	42 5
Heptane	74	Benzene	0	Isoamylbenzene	35 4
Octane	96	Toluene	0	Cetylbenzene	100 6
Decane	102	Ethylbenzene	0	Styrene	0
Pentene	0	*o* Xylene	0	Phenylacetylene	0
Hexene	0	Propylbenzene	0	Methylnaphthalene	29 3
Heptene	0	Cumene	0	Pinene	28 2
Cetene	56 4	Pseudocumene	0	Limonene	14 2
Heptyne	0	Mesitylene	0 6	Terpinene	0

After noting that bacterial cells dried on filter paper were killed more readily by petroleum ether than moist cells of the same species, Baier (3) concluded that the germicidal and bacteriostatic effectiveness of petroleum fractions is a function of their solubility in water Heavy fractions such as paraffin oil were not injurious to bacteria *E coli* was more susceptible to low boiling point fractions than were raw cultures of bacteria in mud or pure cultures of various spore formers Bacteria in nutrient media not killed by exposure to petroleum or its products tended to become increasingly more tolerant, probably through adaptation or the destruction of the bactericidal components (3)

According to Jentsch (78), cyclic hydrocarbons are the only constituents of natural gas which are harmful to bacteria Facultative anaerobes were found to grow in an atmosphere of illuminating gas but not as well as in an atmosphere of nitrogen or hydrogen From his studies on the antiseptic and growth-retarding

actions of cyclic hydrocarbons on bacteria, Van de Velde (161) found that cyclohexane, benzene and toluene had relatively little effect Mesitylene and xylenes were much more effective antiseptics, *o*-xylene being more active than *m*-xylene The antiseptic property of such compounds was found to be roughly parallel to the ease with which they can reduce $KMnO_4$ The microbial oxidation of low concentrations of benzene, toluene and xylene has been reported by many workers (37, 52, 75, 91, 116, 131, 144, 164, 165, 181)

Species of *Penicillium*, *Mucor*, *Torula*, yeasts and bacteria which attacked benzoic acid were found in humus by Perrier (102), who described four varieties of *Bacillus benzoicus* The latter organisms grew in a mineral salts solution enriched with 2% potassium benzoate Phenol and salicylic acid were decomposed more slowly than benzoic acid Observations on the selective action and microbial utilization of phenol, cresol, naphthalene and other aromatic compounds have been discussed in a preceding section (18, 52, 73, 91, 136)

Fowler *et al* (39) isolated from sewage an organism, *Flavobacterium helvolus*, which destroyed 0 1 per cent phenol in mineral salts solution Gray and Thornton (52) noted that phenol in concentrations as high as 0 1% was utilized by *Bacillus closteroides*, *Pseudomonas rathonis* and *Proactinomyces agrestis*, but 0 15% phenol was inhibitory and 0 2% was lethal for these organisms Certain sewage bacteria studied by Kalabina and Rogovskaya (81) utilized phenol as a source of energy in concentrations as high as 0 3% The optimum concentration of phenol for phenol-decomposing organisms was 0 05 to 0 1%, under which conditions sewage bacteria destroyed an average of 57 mg of phenol per day

The following new species of soil organisms which could tolerate or grow in the presence of 0 38% phenol have been described (8) *Bacillus asterosporus*, *B balcanis*, *B catenulatus*, *B exilis*, *B globifer*, *B jubatus*, *B nigrescens*, *B oehensis* and *B phenolphilos* The tolerance of 96 other species of soil bacteria was reported, of which 46 could grow in the presence of 0 1% phenol According to Bartels (8), many soil bacteria produce phenol during the decomposition of proteins

In all one-gram samples of soil examined by Vigier (163), microörganisms were found which tolerated 0 2% phenol in mineral solutions A yeast-fungus was isolated which utilized phenol as a sole source of energy at concentrations as high as 0 085% Optimum growth occurred in mineral media containing from 0 0265 to 0 053% phenol Certain thermophilic bacteria readily oxidized 0 1% phenol in sewage from coke-benzene plants (33) These bacteria, which grew well at 60°, were less active in waste waters containing 0 2% phenol, and 0 4% phenol stopped their growth

Most of the ten lake-mud strains of *Micromonospora* studied by Erikson (37) grew in mineral solutions containing as the only source of energy 0 1% of phenol, toluene, naphthalene, paradichlorobenzene, resorcinol, *m*-cresol, β-naphthol, picric acid, trinitroresorcinol or cholesterol Concentrations of toluene or xylene exceeding 0 01% were injurious to *Bacillus hexacarbovorum* (131) The work of Reed and Rice (105) suggests that the germicidal effect of hydrocarbons may be a function of the lipid content and polarity or wettability of the particular bacterium under consideration

Ethylene has been found to stimulate the respiration of molds, but to retard their growth or multiplication (162)

Insecticidal emulsions containing more than 1% of petroleum oils were found by Young (168) to be injurious to certain plants, although there was considerable difference in the toxicity of different oils and kerosenes used for controlling insect pests on growing fruit and vegetables The relatively rapid disappearance of hydrocarbons from the leaves of plants was attributed partly to evaporation and partly to absorption The lighter fractions of kerosenes penetrated potato leaves within 0 5 to 10 seconds, but mostly evaporated from the leaves within 1 to 24 hours In contrast, lubricating oils penetrated potato leaves within 1 second to 50 minutes and evaporated very slowly or not at all Sections showed that the oils passed from the potato leaves through the stems and into the tubers Petroleum oils were also shown to be absorbed by turnip, rutabaga, cucumber, squash and onion leaves It is an unexplored possibility that much of the oil left on the leaves of plants undergoes microbial decomposition

Young (167) proposed the use of fungi and bacteria to predict the effect of petroleum oils on apple leaves after noting that the toxicity of oils for apple leaves was about the same as for *Rhizopus nigricans*, *Mucor glomerula*, *Helminthosporium sativum*, *Alternaria tenuis*, *Fusarium sp*, *Aspergillus sp*, *Chromobacterium violaceum*, *Serratia marcescens*, *Sarcina aurantiaca* and *Bacillus subtilis* Mold mycelia and bacterial colonies continued to grow on nutrient agar under layers of non-toxic oils 5 cm in thickness Oils containing more than 11% of sulphonatable residue were more toxic than the less sulphonatable oils Hyaline, nearly unsulphonatable petroleum oils were found to be useful for preserving the appearance of many kinds of fungi and bacteria in cultures Confirming the observations of Parish (101), Birkhaug (15) found that cultures of delicate bacteria remained viable in media under layers of liquid paraffin for from *10 to 24* weeks The beneficial effects of the oil were attributed to protection against drying and the harmful action of oxygen

SUMMARY AND CONCLUSIONS

All kinds of gaseous, liquid and solid hydrocarbons in the aliphatic, olefinic, aromatic or naphthenic series appear to be susceptible to oxidation by micro organisms, provided the hydrocarbons are properly dispersed

Nearly a hundred species of bacteria, yeasts and molds have been shown to be endowed with the ability to attack hydrocarbons Such organisms grow in simple mineral media enriched with hydrocarbons as the sole source of energy The presence of free oxygen is generally essential, although nitrate or sulfate serve as hydrogen acceptors for some hydrocarbon oxidizers

Microbial multiplication, oxygen consumption, nitrate or sulfate reduction, modification of hydrocarbons and the formation of various metabolic products have been employed as criteria of the utilization of hydrocarbons The microbial utilization of hydrocarbons has been observed at temperatures ranging from 0° to 60° Being relatively insoluble and immiscible in water, most hydrocarbons are rendered more susceptible to attack when dispersed throughout mineral media by adsorption on inert solids or by emulsification

CO_2 is the principal product resulting from the microbial dissimilation of hydrocarbons Cell substance, organic acids, alcohols, unsaturated compounds and other substances have also been reported

In general, aliphatic hydrocarbons are oxidized more readily than aromatic or naphthenic compounds Within certain limits, long-chain hydrocarbons are attacked more readily than similar compounds of small molecular weight The addition of aliphatic side-chains increases the susceptibility of cyclic compounds to microbial attack

Hydrocarbon-oxidizing microörganisms are widely distributed in soil, water and recent marine sediments They are particularly abundant in oil-soaked soil and water over which petroleum products are stored Characterized as being able to assimilate one or more kinds of hydrocarbons are 14 species of *Actinomyces*, 13 *Pseudomonas*, 10 *Proactinomyces*, 10 *Mycobacterium*, 9 *Bacillus*, 7 *Bacterium*, 7 *Micrococcus*, 6 *Aspergillus*, 5 *Corynebacterium*, 3 *Vibrio*, 2 *Achromobacterium*, several unidentified strains of *Micromonospora* and *Penicillium* and one or more strains of *Botrytis*, *Debaromyces*, *Desulfovibrio*, *Endomyces*, *Escherichia*, *Gaffkya*, *Hansenula*, *Macromonospora*, *Methanomonas*, *Mycoplana*, *Serratia*, *Spirillum*, *Torula* and *Torulopsis*

Although certain crude oils are bacteriostatic, samples of others are destroyed or otherwise modified by microörganisms Microbial activity is believed to have played an important rôle in determining the properties of petroleum deposits, a problem which merits much more attention

Petroleum products stored in contact with water for prolonged periods may be discolored, emulsified or otherwise altered by hydrocarbon-oxidizing microorganisms Coolants and certain kinds of pharmaceuticals containing hydrocarbons and moisture may be adversely affected by microbial activity Natural and most kinds of synthetic rubber are susceptible to microbial oxidation

Microörganisms in soil oxidize methane and other hydrocarbons of biological origin Applications of crude oil to soil result in increased microbial populations and, in many cases, improve soil fertility Even "antiseptic" hydrocarbons such as benzene, toluene, xylene, naphthalene and related compounds are destroyed by microörganisms in soil Phenol, cresols, naphthols, chlorobenzenes, nitrobenzenes, benzoic acid, salicylic acid and resorcinol in low concentrations are attacked by soil organisms The microbial assimilation of phenol in concentrations as high as 0 3% has been demonstrated

Bacteria which oxidize volatile hydrocarbons may provide clues to the location of subterranean deposits of petroleum

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AN ORIENTATION WITH RESPECT TO MEMBERS OF THE GENUS BACILLUS PATHOGENIC FOR INSECTS[1]

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The field of insect pathology is plagued with a confused taxonomic and nomenclatorial situation as concerns those members of the genus *Bacillus* which cause infections in insects As has been pointed out before (Steinhaus, 1942, 1946), there are several reasons for this unfortunate state of affairs One of these reasons has been the frequent lack of understanding of basic rules of nomenclature and systematic arrangement, another has been the absence, until recent years, of a satisfactory system of bacterial systematics to guide even the specialists in this genus Even the current literature covering the field of insect pathology is filled with bacterial names which are entirely inappropriate and incorrect As yet, no serious attempt has been made to segregate those species from the genus *Bacillus* which do not belong in this category Nor has any precise opinion been published as to which of the insect pathogens are correctly named and which are valid species of the genus To assist in doing these two things is the purpose of the present paper

To be sure, the ideal way of undertaking this task would be to study cultures of each of the so-called species in question and to build upon the information thus gained To anyone who gives the matter some thought, however, it is immediately obvious that such a study is impossible at the present time since certainly not more than 10 per cent of the species in the groups concerned (and reported as causing diseases of insects) are available in pure cultures Nevertheless, a considerable amount of underbrush may be cleared away by a thorough and critical study of the literature and recent observations Even then the procedure is a difficult one since many of the descriptions, when such exist, are so woefully inadequate that they do not even indicate whether or not the so-called species of *Bacillus* forms spores In such cases, deductions have to be made from other characteristics that may be given Since the basis on which the genus *Bacillus* now stands appears to be distinct, sound, and no doubt reasonably permanent, there is no good reason why obviously flagrant practices as regards its nomenclature should be tolerated any longer These statements apply to all groups in the genus *Bacillus* but for our purposes here we shall be concerned with only those members which have been reported as parasitic, pathogenic or probably pathogenic for insects

In this paper the genus *Bacillus* referred to is *Bacillus* Cohn (1872, Beiträge z Biol d Pflanzen, **1**, Heft 2, 175), which in the 5th edition of *Bergey's Manual of Determinative Bacteriology* is described as follows

Rod shaped bacteria, sometimes in chains Aerobic Non motile or motile by means of

[1] Contribution from the Laboratory of Insect Pathology, Division of Beneficial Insect Investigations, College of Agriculture, University of California

peritrichous flagella Endospores formed Generally Gram positive Chemo-heterotrophic, oxidizing various organic compounds From Latin *bacillum*, a small stick

The internationally accepted (Jour Bact , 1937, 33, 445) type species is *Bacillus subtilis* Cohn *emend* Prazmowski

On the basis of the action of the International Committee on Bacteriological Nomenclature (Cent f Bakt , II Abt , 1934, 92, 481) which agreed that *Bacillus* be defined so as to exclude bacterial species which do not form endospores, the following alleged species, which have not been described as forming endospores, may be eliminated from the genus at the outset

Bacillus agilis Mattes (1927, Sitz Ges Bedf Natur zu Marburg, 62, 406) [1]
Bacillus agrotidis typhoides Pospelov (1927, Rept Bur Appl Ent , 3, 8)
Bacillus apisepticus Burnside (1928, J Econ Ent , 21, 379)
Bacillus aureus (as used by Eckstein, 1894, Zeit f Forst- u Jagd , 26, 9)
Bacillus barbitistes Statelov (1932, Mitt Bulg Ent Ges , 7, 56)
Bacillus bombycis Chatton (1913, C R Acad Sci , Paris, 156, 1708)
Bacillus bombycis non liquefaciens Paillot (1922, Ann Épiphyt , 8, 131)
Bacillus cajae auct = *Coccobacillus cajae* Picard and Blanc (1913, C R Acad Sci , 156, 1334
Bacillus cleoni Picard (1919, Bull Soc d'Etude Vulgar Zool Agr , 12, 134)
Bacillus coerulus (as used by Eckstein, 1894, Zeit F Forst- u Jagd , 26, 14)
Bacillus coli (as used by Sawamura, 1906, Tokyo Imp Univ Coll Agr Bull , 7, 105) = *Escherichia coli* (Migula) Castellani and Chalmers (1919, Manual of Trop Med , p 941)
Bacillus cubonianus Macchiati (1891 [1892], Malpighia, 5, 289)
Bacillus entomotoxicon Duggar (1896, Bull Illinois State Lab Nat Hist , 4, 371)
Bacillus ferrugenus (as used by Sawamura, 1906, Tokyo Imp Univ Coll Agr Bull , 7, 105)
Bacillus flavus (as used by Eckstein, 1894, Zeit f Forst- u Jagd , 26, 12)
Bacillus foetidus (as used by Eckstein, 1894, Zeit f Forst- u Jagd , 26, 12)
Bacillus fuchsinus (as used by Sawamura, 1906, Tokyo Imp Univ Coll Agr Bull , 7, 105) = *Serratia fuchsina* (Boekhout and DeVries) Bergey et al (1923, Bergey et al , Manual, 1st ed , 91)
Bacillus gortynae Paillot (1913, C R Acad Sci , Paris, 157, 611)
Bacillus lanceolatus (as used by Mattes, 1927, Sitz, Ges Bedf Natur zu Marburg, 62, 406)
Bacillus leptinotarse White (1928, Proc Ent Soc Wash , 30, 71)
Bacillus lineatus (as used by Eckstein, 1894, Zeit f Forst- u Jagd , 26, 17)
Bacillus liparis Paillot (1917, C R Acad Sci , 164, 527, also, 1922, Ann Épiphyt , 8, 123)
Bacillus lutzae Brown (1927, Am Museum Novitates, No 251, p 8 However, see also Brown and Heffron, 1929, Science, 69, 198)
Bacillus lymantriae Picard and Blanc (1913, C R Acad Sci , Paris, 157, 79) = *Bacillus lymantriae* α Paillot (1919, C R Acad Sci , Paris, 168, 258) = *Coccobacillus lymantriae* (of some authors)
Bacillus lymantriae β Paillot (1919, C R Acad Sci , Paris, 168, 258, also, 1922, Ann. Épiphyt , 8, 122)
Bacillus lymantricola adiposus Paillot (1919, C R Acad Sci , Paris, 168, 258, also, 1922, Ann Épiphyt , 8, 121)
Bacillus melolonthae Chatton (1913, C R Acad Sci , Paris, 156, 1708)

[1] In most cases, the page number refers to the page of the article on which the name of the species is first given

Bacillus melolonthae liquefaciens Paillot (1916, C R Acad Sci , Paris, 163, 533) = *Bacillus melolonthae liquefaciens* α Paillot (1922, Ann Épiphyt , 8, 108) and strains β and γ Paillot (1918, C R Acad Sci , Paris, 167, 1046, also, 1922, Ann Épiphyt , 8, 109, 110)
Bacillus melolonthae non liquefaciens α (and strains β and γ) Paillot (1916, C R Acad Sci , Paris, 163, 533), strain δ Paillot (1918, C R Acad Sci , 167, 1047), and strain ε Paillot (1922, Ann Épiphyt , 8, 114, see also pp 111–114)
Bacillus minimus (as used by Eckstein, 1894, Zeit f Forst- u Jagd , 26, 16)
Bacillus neurotomae Paillot (1924, C R Acad Sci , Paris, 178, 247)
Bacillus noctuarum White (1923, J Agr Res , 26, 488)
Bacillus paratyphi alvei Bahr (1919, Skand Veterin Tidsk , 9, 25) = *Salmonella schottmülleri* var *alvei* Hauduroy *et al* (1937, Dict d Bact Path , 469)
Bacillus pectinophorae White and Nobel (1936 J Econ Ent , 29, 123)
Bacillus pediculi Arkwright and Bacot (1921, Parasitol , 13, 26)
Bacillus pieris agilis Paillot (1919, C R Acad Sci , Paris, 168, 477, also 1922, Ann Épiphyt , 8, 128)
Bacillus pieris fluorescens Paillot (1919, C R Acad Sci , Paris, 168, 477, also, 1922, Ann Épiphyt , 8, 124)
Bacillus pieris liquefaciens Paillot (1919, C R Acad Sci , Paris, 168, 477), strain α Paillot (1922, Ann Épiphyt , 8, 125), and strain β Paillot (1922, Ann Épiphyt , 8, 126)
Bacillus pieris non liquefaciens α (and strain β) Paillot (1919, C R Acad Sci , 168, 477, also Ann Épiphyt , 8, 126, 127)
Bacillus poncei Glaser (1918, Ann Ent Soc Am , 11, 23)
Bacillus proteidis Paillot (1922, Ann Épiphyt , 8, 130)
Bacillus pyocyaneus (as used by Sawamura, 1906, Tokyo Imp Univ Coll Agr Bull , 7, 105) = *Pseudomonas aeruginosa* (Schroeter) Migula (1895, in Engler and Prantl, Die Natürlichen Pflanzenfamilien, 1, 29)
Bacillus pyrameis (strains I and II) Paillot (1913, C R Acad Sci , Paris, 157, 611)
Bacillus sphingidis White (1923, J Agr Res , 26, 479)
Bacillus tingens (as used by Eckstein, 1894, Zeit f Forst- und Jagd , 26, 10)

Certain of the above-listed species may, on the basis of their published descriptions or characterizations, be more or less definitely assigned to other genera or be placed in synonomy with existing valid species The majority, however, need considerably more cultural study before this can be done In such cases, it may be advisable in the meantime to assign most of them temporarily to the genus *Bacterium* which is used to cover species of nonsporeforming, rod-shaped bacteria whose position in the system of classification is not definitely established (*Bergey's Manual*, 5th ed , p 590) Unless further study or the accumulation of additional information should show any of these bacteria to be capable of forming spores, they certainly do not belong to the genus *Bacillus* Cohn and should not bear this generic name

Concerning certain species mentioned in the literature on infections in insects, it has been impossible to determine from the information at hand whether or not they are capable of forming spores They are

Bacillus alveolaris (as used by Ksenjoposky, 1916, Rept Volhynia Entomol Bur for 1915, 24 pp)
Bacillus butlerorii Serbinov (1912, Messager de la Société Russe d'Apiculture, No 3)
Bacillus cleonusum Beltiukova and Romanevich (1940, Mikro Zhurnal, 7, 131)
Bacillus fluorescens liquefaciens Flügge (as used by Pospelov, 1927, Repts on Appl Ent , 3, 1)

Bacillus fluorescens non liquefaciens (*putidus* Flügge) (as used by Pospelov, Repts on Appl Ent, 3, 3, 9)
Bacillus gigas van der Goot (1915, Med Proefstation voor de Java Suikerindust, pt 5, No 10, 284)
Bacillus rubefaciens (as used by Sawamura, 1906, Tokyo Imp Univ Coll Agr Bull, 7, 105)
Bacillus salutaris Metchnikoff (1879, Ueber die Krankheiten des Getreidekäfers [*Anisoplia austriaca*] Odessa)
Bacillus septicaemiae lophyri Shiperovich (1925, Protect Plants Ukraine, pt 3-4, 41)

The following list includes those alleged species, without regard for their validity or synonomy, which have been characterized as forming spores, and accordingly may be considered as belonging to the genus *Bacillus*

Bacillus agilis larvae Toumanoff (1927, Bull Soc Cent de Méd Vétér, 80, 367) (= *Bacillus agilis* Hauduroy et al, but not *Bacillus agilis* Mattes)
Bacillus alvei Cheshire and Cheyne (1885, J Roy Micro Soc, Ser 2, 5, 581) (Includes *Bacillus pluton* White, *a nonsporeforming phase*)
Bacillus bombycis auct (Sometimes referred to as "*Bacillus bombycis* Pasteur" Name taken from *Bacillus bambycis* Macchiati, 1891, Staz Sper Agr Ital, 20, 118)
Bacillus bombycoides Paillot (1942, C R Acad Agr, France, 28, 158)
Bacillus bombysepticus Hartman (1931, Lingnan Sci J, 10, 280)
Bacillus brandenburgiensis Maassen (1906, Mitt Kaisl Biol Anst f Land- u Forstwirtsch, 2, 28)
Bacillus burri (as used by Cowan, 1911, Brit Beekeeper's Guide Book, 226 pp)
Bacillus "C" Sokoloff and Klotz (1942, Phytopath, 32, 187)
Bacillus cereus Frankland and Frankland (1887, Philosoph Trans Roy Soc London, 178, B, 279)
Bacillus ellenbachi (as used by Sawamura, 1906, Tokyo Imp Univ Coll Agr Bull, 7, 105 Possibly *Bacillus ellenbachensis* Gottheil)
Bacillus gryllotalpae Metalnikov and Meng (1935, C R Acad Sci, Paris, 201, 367)
Bacillus hoplasternus Paillot (1916, C R Acad Sci, Paris, 163, 774, also 1919, Ann Inst Pasteur, 33, 403, also 1922, Ann Épiphyt, 8, 115)
Bacillus intrapallans Forbes (1886, Bull Illinois State Lab Natur Hist, Art IV, pp 283, 288, 297)
Bacillus larvae White (1905, Thesis, Cornell Univ, Ithaca, N Y also, 1920, U S D A Bull 809, p 13)
Bacillus lastiocampa Brown (1927, Am Museum Novitates, No 251, pp 1 and 7)
Bacillus laterosporus Laubach (1916, J Bact, 1, 511) = *Bacillus orpheus* White (1912, U S Dept Agr, Bur Ent, Cir 157, p 3, also McCray, 1917, J Agr Res, 8, 410)
Bacillus lentimorbus Dutky (1940, J Agr Res, 61, 57)
Bacillus megatherium De Bary (1884, Vergleichende Morph u Biol d Pilze, p 499)
Bacillus megatherium bombycis Sawamura (1905, Tokyo Imp Univ Coll Agr Bull, 6, 375)
Bacillus mesentericus Trevisan (1886, Die Mikroorganismen, p 321)
Bacillus milii Howard (1900, Gleanings in Bee Culture, 28, 122)
Bacillus manachae (von Tubeuf) Eckstein (von Tubeuf, 1892, Forstl Naturwissensch Zeit, 1, 41 Eckstein, 1894, Zeit f Forst- u Jagd, 26, 6) (According to Eckstein, *Bacillus monachae* = *Bacillus Hafmann* = *Bacillus* "B")
Bacillus mycoides Flügge (1886, Die Mikroorganismen, p 324)
Bacillus orpheus White (see *Bacillus laterosporus*)
Bacillus para alvei Burnside (1932, Am Bee J, 72, 433)
Bacillus popilliae Dutky (1940, J Agr Res, 61, 57)

Bacillus septicus insectorum Krassilstschik (1893, Memoires de la Soc Zool , France, 6, 250)
Bacillus similis (as used by Eckstein, 1894, Zeit f Forst- u Jagd , 26 11)
Bacillus sotto auct (referred to as "Ischivata's sudden death bacillus," e g , see Sawamura, 1905, Tokyo Imp Univ Coll Agr Bull , 6 375)
Bacillus spermatozoides (as used by Eckstein, 1894, Zeit f Forst- u Jagd , 26, 13)
Bacillus subtilis Cohn *emend* Prazmowski (Cohn, Beiträge z Biol d Pflanzen, 1872, 1, Heft 2, 175, 1875, Heft 3, 188, 1876, Heft 2, 249 Prazmowski, 1880, Inaug Diss , Leipzig)
Bacillus tenax (as used by Eckstein, 1894, Zeit f Forst- u Jagd , 26, 14)
Bacillus thoracis Howard (1900, Gleanings in Bee Culture, 28, 122)
Bacillus thuringiensis Berliner (1915, Zeit f Angew Ent , 2, 29)
Bacillus tracheitis sive [or] *graphitosis* Krassilstschik (1893, Mem de la Soc Zool France, 6, 250)
Bacillus "X" White (1904, N Y Dept Agr , 11th Ann Rept , p 103) = *Bacillus larvae* White

Some sporeforming bacteria pathogenic for insects have been assigned the generic name *Bacterium* by their describers Since these are sporogenic and aerobic, however, the writer feels that if the species are valid, he is justified in recommending that new combinations be made of each of them and that they be placed in the genus *Bacillus*, although in so doing he does not necessarily recognize them to be distinct and valid species The following are the names concerned

Bacterium canadensis Chorine (1929, Internat Corn Borer Invest , Sci Rept , 2, 39, also, 1929, Ann Inst Pasteur, 43, 1658)
Bacterium cazaubon (two strains) Metalnikov, Ermolaev, and Skobaltzyn (1930, Internat Corn Borer Invest , Sci Repts , 3, 30)
Bacterium christiei Chorine (1929, Internat Corn Borer Invest , Sci Repts , 2, 46, also, 1929, Ann Inst Pasteur, 43, 1666)
Bacterium ephestiae (two strains) Metalnikov and Chorine (1929, Ann Inst Pasteur, 43, 1393) = *Bacillus thuringiensis* Berliner
Bacterium galleriae (two strains) Metalnikov (1922, C R Acad Sci , Paris, 175, 68, also, Kitajima and Metalnikov, 1923, C R Soc Biol , 88, 477)
Bacterium italicum Metalnikov, Ermolaev, and Skobaltzyn (1930, Internat Corn Borer Invest , Sci Repts , 3, 30)
Bacterium ontariori Chorine (1929, Internat Corn Borer Invest , Sci Repts , 2, 44, also, 1929, Ann Inst Pasteur, 43, 1664)
Bacterium pyrenei (two strains) Metalnikov, Ermolaev, and Skobaltzyn (1930, Internat Corn Borer Invest , Sci Repts , 3, 28)

It will be noted that a few of the species listed are well-known saprophytic bacteria and are not entomogenous except as they have occasionally been found causing infections in insects or have been inoculated into insects for experimental purposes Certain others do appear to be almost strictly entomogenous in their habitat Still others have been described so inadequately or have not been characterized in such a way as to permit definite identification that for the time being at least, their names are practically meaningless so far as being distinct species is concerned In fact, it is not likely that the systematic standing of many of this latter group will ever be verified and for all practical purposes most of them may be considered as *nomina nuda*

Those entomogenous species from the foregoing lists which may find acceptance as valid and distinct species of the genus *Bacillus* include the following

Bacillus alvei Cheshire and Cheyne
Bacillus bombycis auct
Bacillus larvae White
Bacillus laterosporus Laubach (=*Bacillus orpheus* White)
Bacillus lentimorbus Dutky
Bacillus popilliae Dutky

Those entomogenous species which may be distinct and valid but which have had insufficient comparative work done on them to ascertain this fact, include

Bacillus bombysepticus Hartman
Bacillus bombycoides Paillot
Bacillus hoplosternus Paillot
Bacillus lasiocampa Brown

EXPLANATORY DISCUSSION

The listings given on the foregoing pages are not entirely self-explanatory as to the taxonomic and nomenclatorial status of all the species concerned The principal points of some of these aspects are briefly analyzed in the discussion which follows

BACILLUS ALVEI In 1885 Cheshire and Cheyne described *Bacillus alvei* as the cause of European foulbrood of the honeybee, *Apis mellifera* L Since then, considerable differences of opinion have been expressed concerning the etiology of this disease and the true status of *Bacillus alvei* Maassen (1907, 1908) believed that a combination of *Bacillus alvei* and an organism he named *Streptococcus apis* were necessary to cause the foulbrood In 1908, White observed a bacterium, which he referred to as bacillus "Y", and which would not grow on the usual artificial media In 1912 he considered this nonsporulating organism to be the exciting cause of European foulbrood and gave it the name *Bacillus pluton* White maintained this position in his comprehensive report on this disease in 1920, considering *Bacillus alvei*, *Streptococcus apis*, *Bacterium (Achromobacter) eurydice* White, and *Bacillus orpheus* White to be secondary invaders

For several years following the work of White, the etiological rôle of *Bacillus pluton* was accepted with some workers (e g , Sturtevant, 1925) pointing out that even in a secondary rôle, *Bacillus alvei* probably had a marked influence upon the course of the disease Then, in 1928, Lochhead showed that *Bacillus alvei*, when grown for several weeks on sugar-containing media, possessed a coccoid stage which appeared similar to *Bacillus pluton* Lochhead questioned whether *Bacillus pluton*, as a separate species, could be said to exist at all, since it has never been known to be obtained in pure culture, Wharton's (1928) report on its cultivation having been discounted by Lochhead. Wharton states, however, that "cultures of *B pluton* have been observed to change to *B alvei* form resembling biologically the *B alvei* isolated from infected larvae"

In 1934, Burnside published an account of his studies on the bacteria associated with European foulbrood in which he asserted that no evidence has been obtained

which satisfactorily explains the etiology of this disease in bees He noted that several morphologically different bacterial forms are more or less constantly present in honey bee larvae sick or dead of European foulbrood, these forms are absent in larvae sick or dead of other causes Of particular significance, as regards the present discussion, is Burnside's observation that "*Bacillus alvei* is capable of morphological, cultural, and biological transformation and is also capable of stabilization, at least temporarily, as a sporogenic rod, an asporogenic rod resembling *Bacterium curydice*, or a coccoid resembling *Bacillus pluton*" As to the identity of *Streptococcus apis* and *Bacillus pluton*, Burnside appears to be quite certain He further suggests that *Bacillus pluton* and *Streptococcus apis* are variants, or stages in the life history of *Bacillus alvei* Among the several reasons for this belief, he cites the occurrence of variants resembling *Bacillus pluton* in pure cultures of *Bacillus alvei* and by the apparent origin on rare occasions of sporogenic *Bacillus alvei* in cultures of *Streptococcus apis* Tarr (1935) suggests that there may be several distinct strains of *Bacillus alvei* which may be differentiated on the basis of their fermentative powers

Several other theories on the etiology of European foulbrood have been presented in the literature but none of these are based on direct proof Nearly all recent evidence tends to support Burnside's concept and until conclusive data to the contrary are put forward, there seems to be no good reason why *Bacillus alvei* is not a species in good standing and the causative agent of European foulbrood If *Bacillus pluton* is in fact a separate and distinct species it should be removed from the genus *Bacillus* since it is not sporogenic

Neide's (1904) *Bacillus alvei* Krompecher may be considered synonymous to *Bacillus alvei* Cheshire and Cheyne

Observations by Clark (1939) and by Nathan R Smith (personal communication) strongly suggest that *Bacillus para-alvei* is essentially the same as *Bacillus alvei* and that they may be considered to be the same species

BACILLUS BOMBYCIS According to the modern theories concerning flacherie (flachery or flaccidity) in the silkworm, *Bombyx mori* (L), an ultravirus is the true cause of this disease in which bacteria are important secondary invaders When *Streptococcus bombycis* is the secondary invader the syndrome known as *gattine* results, when the secondary invader is *Bacillus bombycis* auct , the condition known as true flacherie or the flacherie of Pasteur is the consequence Therefore, even though *Bacillus bombycis* is not the exciting cause of the disease, nevertheless, under certain conditions, it may be said to have pathogenic properties and very likely is an important factor in the course of the disease

The name *Bacillus bombycis* is surrounded with several nomenclatorial vagaries which confuse the picture considerably Unfortunately, Pasteur (1870), who discovered the organism, neither named or precisely described it He characterized it as being "des vibrions, souvent très-agiles, avec ou sans noyaux brillants dans leur intérieur" In 1891 Macchiati gave the name *Bacillus bombycis* to a sporeforming organism he found in larva, pupa, and adult of the silkworm There are several reasons for believing that Macchiati's organism is not the same as Pasteur's "vibrion à noyau," two of which have been pointed out by Paillot

(1930) (1) Pasteur's bacillus is not cultivable on ordinary bacteriological media, whereas Macchiati apparently had no difficulty in thus growing his organism, (2) Pasteur's bacillus is readily decolorized in using Gram's method of staining, while Macchiati's organism apparently is not According to Paillot, these two criteria would also serve to differentiate Pasteur's organism from Ishiwata's *Bacillus sotto*, a bacterium found in association with flacherie in Japan and similar to another sporeformer found in diseased silkworms in France Also thus eliminated would be the possibility suggested by Sawamura (1905) that the organism with which we are concerned is a strain of *Bacillus megatherium*

According to accepted procedures of nomenclature, Macchiati's original use of the name *Bacillus bombycis* for the cultivable sporeformer he isolated would preclude its use for Pasteur's bacillus In light of the dissimilarity between Macchiati's and Pasteur's organisms, however, Paillot (1930) has seen fit to retain the name *Bacillus bombycis* for Pasteur's bacillus and to suggest that another name be given to the species isolated by the Italian author Thus, throughout most of the recent literature on flacherie the name *Bacillus bombycis* has been used to designate the sporeformer observed by Pasteur and subsequent workers Macchiati's bacillus has had no additional work done on it and a guess might be that it was one of the common, more-or-less saprophytic sporeformers known today Paillot's procedure in using the name *Bacillus bombycis* for Pasteur's organism makes a homonym of the name and is contrary to the accepted rules of nomenclature It therefore becomes a question of accepting it on the basis of common usage or of proposing a new name for it The latter procedure would no doubt be the more correct one but whether a new name would now be accepted by workers in this field is another matter Since nearly all users of the name *Bacillus bombycis* have used it in referring to Pasteur's organism (and Macchiati apparently thought be was dealing with Pasteur's organism), its acceptance in its present form would probably be favored by most authorities

The name *Bacillus bombycis* has been used by Chatton (1913) for a small, nonsporeforming gram-negative rod Paillot (1930) used the name *Bacillus bombycis non liquefaciens* for a nonsporulating bacterium In both of these cases it is clear that the names are not valid since both are antedated by Macchiati's use of the name for the sporeformer

Whether any of the bacteria mentioned in the preceding paragraphs is Joly's (1858) "*Vibrio Aglaiae*," apparently the first bacterium described from diseased silkworms, is uncertain

OTHER SPECIES Although the fifth edition of *Bergey's Manual* does not include a recognition of *Bacillus larvae* White, there is no good reason why it is not entitled to full taxonomic standing[3] *Bacillus larvae* is the cause of American foulbrood in the honeybee, *Apis mellifera* L., and has received a considerable amount of study Synonyms include *Bacillus "X"* White, *Bacillus brandenburgiensis* Maassen and *Bacillus burri* (as used by Cowan)

Bacillus lentimorbus and *Bacillus popilliae*, described by Dutky in 1940,

[3] Dr Robert S Breed has informed the writer that the forthcoming 6th edition of the *Manual* will include a description of *Bacillus larvae*

are species causing the so-called milky diseases of grubs, particularly those of the Japanese beetle, *Popillia japonica* Newman These milky diseases are playing an important rôle in the control of the Japanese beetle in the eastern United States Both bacterial species, particularly *Bacillus popilliae*, have been relatively well studied and are valid and distinct species

Bacillus orpheus was the name given by White (1912) to a sporeforming bacillus found occasionally in bees affected with European foulbrood It, in itself, was not found to be pathogenic for bee larvae and White (1920) considered it to be one of the secondary invaders encountered in the disease Recently Smith, Gordon, and Clark (1946) have presented evidence showing that *Bacillus orpheus* is identical with *Bacillus laterosporus*, originally isolated by Laubach in 1916 from water Although White named the organism in 1912, a description was not published until 1917 (McCray, 1917) In the meantime Laubach's description of *Bacillus laterosporus* appeared, thus invalidating the name *Bacillus orpheus* which Smith *et al*, have suggested should be discarded

A common soil organism, *Bacillus cereus*, has been found as a pathogen for insects on several occasions and there is a good possibility that several of the strains described under other names (e g , *Bacillus ellenbachi*) are in reality of this species Nathan R Smith (personal correspondence) has determined as *Bacillus cereus* the strain of *Bacillus thuringiensis* sent to him by Mattes (1927) Similarly, Smith found a culture of *Bacillus* "C" obtained by the writer from Dr L J Klotz, to be a typical strain of *Bacillus cereus* No doubt other common sporeformers are represented in the list presented on the foregoing pages

Certain species, if valid, are in need of renaming if for no other reason than to place them in binomial form In some cases, the use of hyphens in forming the specific epithet would not help a great deal In other cases, agreeablenames could probably be formed in this manner, e g , *Bacillus septicus-insectorum* comb nov

SUMMARY

This paper is an attempt to assist in segregating from the genus *Bacillus* Cohn those species of bacteria pathogenic for insects which do not form endospores and to express an opinion as to which of the insect pathogens of the group of sporeformers concerned are correctly named (generically), and to indicate which are probably the valid species of the genus

Listed with their original citations are 45 alleged species which have been given the generic name *Bacillus* but which are not sporogenic and which, therefore, should be removed from the genus *Bacillus* Cohn Forty-four names are listed, also with their original citations, of presumably aerobic bacteria which have been characterized as forming spores, and which accordingly may properly be considered as belonging to the genus *Bacillus* Eight of these sporeforming species were originally assigned the generic name *Bacterium* by their describers but correctly belong to the genus *Bacillus* The names of nine species are given

for which it has been impossible, from the information available, to determine whether or not they are capable of forming spores

The nomenclatorial and taxonomic vagaries of certain of the species (e g, *Bacillus alvei* and *Bacillus bombycis*) are discussed in some detail

Since the basis on which the genus *Bacillus* now stands appears to be distinct, sound, and no doubt reasonably permanent, it is recommended that greater care be taken by both microbiologists and entomologists working in this field to avoid the flagrant misuse of the generic name *Bacillus* in the identification and classification of bacteria encountered in their work

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CHEMICAL NATURE AND BIOLOGICAL SPECIFICITY OF THE SUBSTANCE INDUCING TRANSFORMATION OF PNEUMOCOCCAL TYPES[1]

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The development of the science of bacteriology has been marked by notable advances in the study of disease and in the control of industrial and agricultural processes in which microörganisms play a vital rôle For the most part, these advances arose from a study of the general properties of bacteria and of the relationship between them and their environment In recent years, as a result of a closer inspection of the biology of microörganisms, especially as related to biology in general, there has been an increasing array of new applications of bacteriological material and methods to a variety of problems This trend toward broadening the scope and significance of bacteriology is exemplified by such developments as the use of bacteria as a source of specific enzymes, as tools for the quantitative assay of numerous vitamins and amino acids, as material for research in genetics, as producers of antibiotic agents, and as a guide to the understanding of certain biochemical reactions of higher organisms Accompanying this trend has been an increasing recognition of the fact that while bacteria have certain unique properties, their metabolic activities and biochemical behavior are strikingly analogous to those of higher organisms The studies to be described on the transformation of pneumococcal types had their origin in an attempt to clarify a phenomenon that appeared to be quite unique and limited to one species of microörganisms However, as information accumulated concerning the more intimate aspects of the problem, it became apparent that in this case also broader principles must be involved and that the results have implications beyond those referable to the biology of the pneumococcus Thus, the present work provides another example of the potential relationship of the knowledge gained from microbiological investigations to problems in other fields of scientific research

The phenomenon of transformation of pneumococcal types was discovered by Griffith in 1928 He found that it was possible to transform a non-encapsulated (R) variant derived from one specific type of pneumococcus into encapsulated (S) cells of a heterologous specific type In the original experiments this was accomplished by injecting into mice a small inoculum of living R pneumococci, along with a large amount of heat-killed cells of an encapsulated strain of a separate type Many mice so treated died of pneumococcal infection and post-mortem culture revealed the presence of virulent, encapsulated pneumococci

[1] Address to the Society of American Bacteriologists by the recipient of the Eli Lilly and Company Award in Bacteriology and Immunology Detroit, Mich , May 24, 1946 The Bureau of Medicine and Surgery of the U S Navy does not necessarily undertake to endorse the views or opinions which are expressed in this paper

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of the same type as that of the heat-killed cells inoculated These experiments were quickly confirmed in several other laboratories (Neufeld and Levinthal, 1928, Reimann, 1929, Dawson, 1930, Baurhenn, 1932), and it was thus established that predictable and type-specific modifications could be produced in pneumococci under the influence of some substance present in heat-killed cells Subsequently, Dawson and Sia (1931) were able to obtain similar transformations *in vitro* by growing an R strain in broth containing anti-R serum and heat-killed encapsulated S cells The next major advance occurred when Alloway (1932, 1933) was successful in separating the active principle from encapsulated cells so that *in vitro* transformation could be carried out with cell-free extracts This work established the fact that the substance responsible for the induction of transformation can be obtained in a soluble form, and it paved the way for attempts to determine its chemical nature The studies to be summarized in this review deal with the purification of the active transforming substance and with the identification and properties of the purified material Work on this problem has been carried out by Dr O T Avery over a period of several years, with the collaboration first of Dr C M MacLeod and later of the present writer

Crude transforming extracts from heat-killed pneumococci are complex mixtures of many constituents of the bacterial cells, and the preliminary approach to purification involved a process of eliminating the inactive components The removal of protein, lipids, ribonucleic acid and capsular and somatic polysaccharides from Type III pneumococcal extracts proved to have no effect on transforming activity Consequently, the method for purifying the Type III transforming substance includes procedures for separating each of these inactive components from the active material the protein by the chloroform method, the capsular polysaccharide by digestion with a specific bacterial enzyme which hydrolyzes it, the somatic polysaccharide by fractional alcohol precipitation, and ribonucleic acid either by enzymatic digestion with ribonuclease or by alcohol fractionation (Avery, MacLeod and McCarty, 1944)

The final product obtained after application of these procedures possesses practically all the activity of the original crude extract It is readily soluble in water or saline and gives clear, colorless solutions that are highly viscous even at relatively low concentrations The material is precipitated by alcohol in the form of a mass of fibrous threads and loses none of its biological activity on repeated precipitation with alcohol Qualitative tests for protein and ribonucleic acid give negative results On the other hand, the diphenylamine reaction for desoxyribonucleic acid (thymus type) is strongly positive, and the results of semi-quantitative determinations by this method indicate that the bulk of purified material must be made up of this type of nucleic acid These results are amply confirmed by the elementary chemical analysis The high phosphorus content of 8 5–9 0 per cent and the nitrogen-phosphorus ratio of 1 67 agree closely with the values ascribed to the sodium salt of nucleic acid of this type Indeed, the nitrogen-phosphorus ratio provides further evidence for the absence of nitrogen-containing impurities such as protein Absorption curves in the ultraviolet region show the high maximum at 2600 Å characteristic of nucleic acids

From these data, it can be concluded that the active material is concentrated in a desoxyribonucleic acid fraction of the pneumococcal cells

Additional evidence concerning the efficacy of purification of the desoxyribonucleic acid fraction is obtainable by serological techniques, since the purified material does not give precipitin reactions when tested with Type III antipneumococcal sera of high titer In view of the high reactivity of both the capsular and somatic polysaccharides and of pneumococcal protein, the results confirm the impression that these serologically active substances have been removed

Analysis of the active desoxyribonucleic acid fraction by electrophoresis and ultracentrifugation reveals that the material is surprisingly homogeneous in both the electrical and centrifugal fields In each case, there is a single, sharp boundary and the transforming property appears to migrate with the visible boundary, indicating that biological activity is associated with the desoxyribonucleic acid

The transforming activity of the purified material is estimated quantitatively by testing the ability of serial dilutions to induce transformation of susceptible R cells into encapsulated Type III pneumococci The tests are carried out in a special serum broth which appears to be optimal for the reaction As little as 0 003 microgram of the desoxyribonucleic acid fraction from Type III pneumococci, representing a final dilution of 1 part in 600,000,000, is capable of causing the transformation

The data which have been briefly summarized demonstrate that a purified desoxyribonucleic acid fraction isolated from Type III pneumococci possesses the property of inducing predictable and type-specific modifications in unencapsulated cells derived from a heterologous type of pneumococcus The change induced is permanent and the transformed cells retain their capsular structure and type-specificity through repeated subculture Furthermore, the active substance can be isolated from transformed cells in amounts far in excess of that originally used to induce the change, indicating that in addition to initiating the process of capsular synthesis, it is itself reduplicated in the cell The question then arises whether the substance displaying these unique properties is actually desoxyribonucleic acid The traditional view stemming from early biochemical studies considers the nucleic acids as more or less invariable in structure, regardless of their occurrence in nature, and there is no indication that they possess the capacity to exert a biologically specific action comparable to that of the transforming substance Consequently, although all the data obtained by chemical, physical and serological techniques indicate strongly that the biological activity of the transforming substance is a property of the desoxyribonucleic acid, further means were sought to obtain more direct and conclusive evidence that the nucleic acid itself and not some other substance, present in small amounts and intimately associated with the nucleic acid, is responsible for transforming activity

Enzymes provide one of the most specific biochemical tools for application to this type of problem When an enzyme is reasonably purified and intelligently employed, its effect on the activity of a biologically active molecule can in some instances supply direct and unambiguous evidence concerning the chemical nature of the latter Numerous examples of this use of enzymes have appeared

in recent years, especially since more purified enzyme preparations of various sorts have become available The bacterial enzyme previously mentioned which hydrolyzes the capsular polysaccharide of pneumococcus Type III was originally isolated by Dubos and Avery (1931) to provide an enzymatic reagent that could be used to confirm the polysaccharide nature of this substance It is of interest that in this case enzyme specificity surpasses the specificity of serological reactions, since the capsular polysaccharide of pneumococcus Type VIII which cross reacts with Type III in serological tests is not attacked by the specific Type III enzyme

Enzymatic techniques were applied relatively early in the course of the attempts to determine the nature of the transforming substance It was found, for example, that crystalline preparations of trypsin, chymotrypsin and ribonuclease have no effect on the biological activity or physical properties of the transforming agent, thus providing further indication that protein and ribonucleic acid do not play a vital rôle in the transforming tests On the other hand, certain crude enzyme preparations, including intestinal phosphatase, pancreatic extracts, dog and rabbit sera, and pneumococcal autolysates, have the capacity of causing rapid and complete loss of transforming activity (Avery, MacLeod, McCarty, 1944) These same enzyme preparations also possess the ability to hydrolyze highly polymeric preparations of desoxyribonucleic acid from animal sources, but because of the crude nature of the enzyme mixtures it is impossible to determine with certainty whether the inactivation of the transforming substance and the hydrolysis of desoxyribonucleic acid are due to one and the same enzyme Obviously, then, the reagent required in order to provide conclusive results must be a purified preparation of the enzyme which attacks desoxyribonucleic acid However, this enzyme, desoxyribonuclease, was not available in purified form, and its isolation and purification were therefore undertaken in our laboratory

Native desoxyribonucleic acid, as prepared from animal tissues by methods designed to avoid partial breakdown, is a highly polymeric substance with a molecular weight of approximately 1,000,000 The molecules are elongated and asymmetrical and, as a result, solutions of the sodium salt have a high degree of structural viscosity Enzymatic hydrolysis of native desoxyribonucleate breaks the molecule down to relatively small polynucleotide units and is accompanied by great loss of viscosity It is therefore convenient to measure the action of desoxyribonuclease by following the fall in viscosity of a solution of calf-thymus sodium desoxyribonucleate when treated with the enzyme under standard conditions This method proved to be sufficiently reliable and reproducible so that it could be employed for the quantitative estimation of the activity of various enzyme fractions obtained in the course of purification procedures

Early in the course of preparative procedures, it was found that desoxyribonuclease requires activation by magnesium ions It displays little or no activity in the absence of the metallic activator Magnesium ion was therefore included in optimal concentration in the routine procedure for measuring the enzyme activity

Although desoxyribonuclease is present in a wide variety of tissues, it occurs in by far the highest concentration in pancreas Separation of the enzyme from the bulk of other pancreatic enzymes was finally accomplished by application of the acid-extract method devised by Kunitz and Northrop (1935) for the isolation of the tryptic enzymes Beef pancreas is extracted with 0 25N H_2SO_4 and the desoxyribonuclease is precipitated by relatively low concentrations of ammonium sulfate which do not throw down appreciable quantities of the other enzymes By further salt fractionation of this material, a desoxyribonuclease preparation is obtained which is free from lipase, phosphatase and ribonuclease activity, and contains only traces of a proteolytic enzyme (McCarty, 1946) The specific activity on desoxyribonucleic acid is very high Under optimal conditions of pH, temperature and magnesium ion concentration, a readily measurable effect on the viscosity of sodium desoxyribonucleate solutions is obtained at enzyme concentrations below 0 01 microgram per ml More than 10,000 times this concentration is required to demonstrate the presence of the traces of proteolytic enzyme which are present as a contaminant It would appear that the degree of purification achieved is sufficient to provide the specific enzymatic reagent that was sought for use in the study of the transforming substance

Desoxyribonuclease is readily soluble in water and quite stable in solution at pH 4 to 5 In contrast to the heat-stable ribonuclease, it is very susceptible to heat inactivation Highly effective inhibition of desoxyribonuclease can be obtained by the use of citrate, which apparently acts by forming a soluble complex with magnesium and thus removing the essential magnesium ion from the environment In addition, desoxyribonuclease is markedly inhibited by serum of rabbits immunized with the enzyme protein

The purified desoxyribonuclease was tested for its effect on the transforming activity of the pneumococcal nucleate under the same experimental conditions that were used in the case of the viscosimetric determinations using animal nucleate (McCarty and Avery, 1946a) Samples of transforming substance from pneumococcus Type III were mixed with varying concentrations of desoxyribonuclease and incubated for 30 minutes At the end of this period, the reaction was stopped by heating at 60°C to inactivate the enzyme, and the mixtures were titered for transforming activity It was found that the transforming substance is completely and irreversibly inactivated at final enzyme concentrations as low as 0 005 microgram per ml Even at an enzyme concentration of 0 001 microgram per ml a definite loss of activity occurs in comparison with untreated control material As in the case of the hydrolysis of animal nucleic acid, citrate inhibits the action of desoxyribonuclease on the transforming substance

The data from enzymatic studies therefore provide strong supporting evidence for the view that the biological activity of the transforming substance is a property of the desoxyribonucleic acid Certainly there can be little doubt that desoxyribonucleic acid must be present in its intact, highly polymerized form, and when all the evidence is considered it appears extremely unlikely that small traces of some other specific substance, such as a protein, could be responsible for the manifestation of the transforming activity

The possibility is therefore suggested that nucleic acids possess a type of biological specificity that has hitherto been unsuspected Nucleic acids are apparently present in all living cells and it will be recalled that the desoxyribose type occurs exclusively in the nucleus A reconsideration of the possible rôle of nucleic acids in vital phenomena is indicated in the light of the observed properties of pneumococcal desoxyribonucleic acid These properties are characterized by two cardinal effects the induction in pneumococci of predictable and heritable modifications and the self-reproduction of the active agent in transformed cells

Indeed, there are certain striking analogies between the biological properties of the transforming substance and those of viruses and genes For example, as in the case of viruses, the transforming agent acts only on susceptible living cells, it is transmissible in series and can subsequently be recovered in amounts far in excess of that originally used as inoculum As in the case of genes, the transforming substance behaves as a heritable unit in that it produces predictable and durable alterations in cellular structure and function and is itself reduplicated in daughter cells through successive generations It intervenes in the metabolism of the R cell, giving rise to the synthesis of a new capsular substance, which in turn endows the cells with distinctive and biologically specific characters not possessed by the parent strain Although the validity of these analogies may be questioned, they serve to underline the possible implications of the phenomenon of transformation in the field of genetics and in virus and cancer research

With respect to the biological specificity of the pneumococcal nucleic acid, it should be pointed out that in all probability only a small portion of the total molecules are endowed with transforming activity A desoxyribonucleic acid fraction can be extracted from unencapsulated R pneumococci which is similar in all respects to the Type III preparations, except that it is wholly inactive in the transforming system It is possible that the nucleic acid of R pneumococci is concerned with innumerable other functions of the bacterial cell in a way similar to that in which capsular development is controlled by the transforming substance The desoxyribonucleic acid from Type III pneumococci would then necessarily comprise not only molecules endowed with transforming activity, but in addition a variety of others which determine structural and metabolic activities possessed in common by both the encapsulated and unencapsulated forms This implies that any given desoxyribonucleic acid preparation represents a complex mixture of a large number of entities of diverse specificity

In addition to enzymatic inactivation of the pneumococcal transforming substance, another form of inactivation has been observed which is of special interest because of its reversibility In the presence of ascorbic acid and traces of cupric ion, at neutral or slightly alkaline reaction, the transforming substance is rendered completely inactive Sulfhydryl compounds, such as cysteine and glutathione, protect the material from this type of inactivation Furthermore, transforming preparations which have been completely inactivated by ascorbic acid can be almost quantitatively reactivated by treatment with glutathione The mecha

nism of the effect is apparently that of a reversible oxidation, and the action of ascorbic acid depends upon its autoxidation with concurrent formation of peroxides, which in turn act on certain reversibly oxidizable, but at present unidentified, groups in the molecule of transforming substance (McCarty, 1945)

In addition to its value in establishing the chemical nature of the transforming substance, the isolation of desoxyribonuclease in purified form provides a valuable tool for the study of desoxyribonucleic acids in several fields of investigation For example, it has been applied to the histochemical study of the occurrence and distribution of desoxyribonucleic acid in normal and cancerous tissues Moreover, the knowledge obtained concerning the properties of the enzyme has contributed further to the problem of pneumococcal transformation by making possible a new method for the isolation of the transforming substance from Type III cells Use of this improved method results in a considerable increase in the yield of active material, and it has been employed successfully in the preparation of purified transforming substance from pneumococci other than those of Type III (McCarty and Avery, 1946b)

Because of the ease with which pneumococci undergo lysis, advantage was taken of this property in the first attempts to obtain the transforming substance in a soluble, cell-free form It was found, however, that active preparations could not be made from lysates, and it seemed likely that enzymatic processes were responsible for destruction of the material during lysis This view is supported by the fact that active material can be extracted from cells which have been heat-killed at 65°C , a temperature known to inactivate most of the intracellular enzymes The studies on desoxyribonuclease and its effect on the transforming substance suggested the possibility that pneumococcal cells contain this enzyme, and that it is responsible for destruction of transforming activity during lysis of the cells Actually, it can be readily demonstrated that pneumococci elaborate a desoxyribonuclease which is similar to the pancreatic desoxyribonuclease in its pH optimum, heat lability, and dependence on the presence of magnesium ion The latter property is important because it provides a means of inhibiting the action of the enzyme by the use of citrate The relation of the inactivation of the transforming substance during lysis to desoxyribonuclease activity was established by showing that in the presence of citrate the transforming substance is released from the cell without coincident destruction By allowing cells to lyse in a citrate solution, five times as much active material can be recovered from Type III cells than by the original method of extracting heat-killed organisms

As mentioned previously, although the initial studies on the chemical nature of the transforming substance were all carried out with Type III pneumococci, the citrate method has provided a means of obtaining good yields of the specific transforming substance from pneumococci of other types Purified material has been prepared from Type II and Type VI pneumococci in the form of desoxyribonucleic acid fractions similar in physical and chemical properties to the Type III material and distinguishable from one another only on the basis of their

specificity in the transforming system Therefore, in the three instances studied, and by inference probably in all other types of pneumococci, the active agent belongs to the same general class of chemical substances

The investigations which have been described give us little insight into the mechanism of the transforming reaction It would be of interest to know the manner in which the specific desoxyribonucleic acid enters a susceptible cell and how it sets in motion the series of biochemical reactions that culminate in the synthesis of the capsular polysaccharide It is possible that some knowledge concerning mechanism may be forthcoming from studies now in progress on the rôle of the factors present in serum which are required in the transformation system The data so far obtained are interpreted as indicating that the serum factors act by altering the surface of the bacterial cell so that the specific desoxy ribonucleic acid is taken up or absorbed

In summary of the present work, it seems possible to conclude that the accumu lated evidence has established beyond reasonable doubt that the active substance responsible for transformation is a specific nucleic acid of the desoxyribose type The isolation of desoxyribonuclease in purified form supplies a specific enzymatic reagent for application not only to this problem, but to a variety of others involv ing desoxyribonucleic acid The question of the vital function and biological specificity of nucleic acids in general deserves reconsideration in the light of the observed properties of the pneumococcal transforming substance

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THE SIGNIFICANCE AND APPLICATIONS OF BACTERIOPHAGE IN BACTERIOLOGICAL AND VIRUS RESEARCH[1]

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Hartman (41) in his recent plea for "the little researcher", remarked "that war does not favor the discovery of new principles in science" War may, however, by forcibly reminding us of the fact that applied research too frequently outstrips pure research, provide a subsequent stimulus to basic investigations No one can foresee the basic discoveries of to-morrow, the predictable belongs to the level of applied research At best we can merely select phenomena which we do not understand, confident that if we investigate them in a true scientific spirit, some useful knowledge will emerge

Advances depend equally on new ideas and new tools or methods Our inanimate tools and materials must be designed and operated, they lack the properties of self-assembly and self-duplication The efficacy and sensitivity of our methods depend upon our skill and knowledge Standard methods are apt to beget standard ideas and may impede progress if used uncritically in the research laboratory This address is based, in part, on the thought that in the investigation of primitive living things like bacteria it may sometimes be more appropriate and more profitable to employ agents endowed with certain attributes of living matter Such agents, the bacteriophages or bacterial viruses, may be regarded and employed as ultramicroscopic technicians

In the past two decades, our knowledge of mammalian viruses has vastly increased and effective vaccines for some have been developed, but the really difficult problems of their control lie before us Some may be solved by empirical methods, expensively, as regards time, materials and labour Others will remain unsolved until we have an adequate knowledge of virus-host cell relationships and virus mutations Humanitarian and economic considerations demand that the direct attack on human and animal virus diseases be not only continued, but reinforced The most effective reinforcement is not necessarily the assignment of more funds and more personnel to particular practical problems but to the extension of our theoretical knowledge and the development of new tools, new methods, and new and more fruitful points of view

Probably the most important and difficult problem which has to be considered in encouraging and in providing for bacteriological and virus research is the problem of achieving the optimum balance between applied and basic research

There is an increasing realization of the value of bacterial viruses as materials for basic study Although phage and susceptible bacterium provide the simplest virus-host cell system for investigation, the mechanisms of adsorption and the

[1] Presidential Address delivered before the Society of American Bacteriologists at its Forty-sixth General Meeting, Detroit, Michigan, May 23, 1946

innumerable patterns of virus-host cell relationships are very far from being simple Many phenomena have been observed with phage and host cell which cannot be satisfactorily explained and the mode of reproduction of the phage remains a mystery We cannot ignore these problems just because they appear to be of no practical interest, of no immediate value at the applied levels of the various branches of our science They are significant, for they emphasize the inadequacies of our knowledge of cellular biochemistry, of bacterial genetics, of the mechanisms of virus reproduction, and of cellular immunity to viruses

There has been such an increased interest recently in bacteriophage that any comments of mine might appear superfluous. Further, the subject is so extensive that I cannot hope to do more than select a few of its aspects which, to me, appear to be of relative importance Indeed, in selecting, I must enter the plea that I regard this as a privileged occasion on which a comprehensive review is not demanded

I am not going to stress controversial matters, because I feel that discussion of these is premature Apropos of the various limited observations to which I shall refer, I would like to tell the story of the inquisitive deep sea fish Like other denizens of the dark abysmal zone, this species was phosphorescent and the study of various patterns of light spots was a necessary part of the education of the young There was, of course, a rule against swimming above a certain level. This had its basis in the limits of adaptability to changing conditions of pressure and temperature The particular species to which I refer was unique in adaptability, however, although this was not realized

One day an independent young fish said, "To heck with the rules," and went off on his own, swimming up and up and up until he reached the surface The sun had set, but there was a new moon in the western sky and the fish, after gazing astonished at this strange phenomenon, hastened back and told his companions The news finally reached the ears of the older fish who said, "Nonsense," and stated that the rule about swimming above a certain level would be enforced Some time later, another fish, thinking that there might be something in the story after all, decided to investigate He swam up and up and up When he reached the surface it was night There was no moon, only a brilliant carpet of stars He looked around, but could see no crescent of light such as the earlier adventurer had seen, and disappointed, he returned He told his friends that it was quite true that there were lights in the upper region, but they were stationary and not very interesting and he couldn't see any trace of the miraculous crescent that his predecessor had seen A little later another fish succumbed to curiosity He, however, reached the surface about noon Recovering himself, after being blinded by the glare of the sun, he hurried back in a state of great excitement and told his friends of the amazing, dazzling light that he had seen The news inevitably reached the elder fishes They felt that this nonsense had gone too far and solemnly held a meeting A commission was formed to enquire into the matter and several days later, or rather nights later, the members proceeded to the surface Unfortunately, the sky was heavily overcast and it was the middle of the night On its return the commission reported that illumination was

definitely restricted to the abysmal zone The fish who had reported strange phenomena outside this region were publicly reprimanded for having disturbed the peace of mind of the community

Bacteriophage initially aroused great interest because of its dramatic lytic effect on bacteria *in vitro* The hope of practical application in human disease sustained investigation for a period When the therapeutic inefficiency of phage became apparent, interest languished, until revived by the work of Andrewes, Elford, Burnet, Asheshov, Schlesinger and others, (see reviews and articles by Burnet (11), Elford (27), Asheshov *et al* (8) and Delbrück (22)) The diversity of the sizes of different phages was established The host specificity of many phages was correlated with the presence of a particular bacterial antigen The neutralization of bacteriophage by specific antiserum was studied extensively There was an increasing recognition of the essential similarity of the phages and animal and plant viruses More recently, the electron microscope has brought the phages within range of visual appreciation It has not only confirmed previous estimates of their sizes, but has surprised us with some morphological revelations

The bacteriophages share the following properties with animal and plant viruses

(*a*) They have a similar size range Some species are as small as 10 mμ, others as large as 75 mμ

(*b*) They reproduce only inside a living host cell

(*c*) Their host range may be wide, or limited to a single species

(*d*) They may destroy the host cell, or may continue to exist within it in a latent state

(*e*) They may extend or change their host specificity by adaptation or mutation

(*f*) The presence of one phage may interfere with infection by another

(*g*) They are antigenic, producing antibodies which neutralize them

These similarities are the basis for recommending phage and susceptible bacterial cell as model material for investigations which may aid progress with mammalian viruses[2] Host specificity and apparent dependence on specific metabolic steps in the bacterial cell are considerations which suggest various ways in which phage may aid studies of the host cell I have not attempted to divide my address into two portions, one devoted to viruses, the other to bacteria It is more convenient to follow a sequence dictated by the phage particle, even if the resulting presentation appears somewhat disjointed

THE BACTERIOPHAGE PARTICLE

Each species of bacteriophage which has been measured has a characteristic particle diameter which is constant within narrow limits Electron micrographs

[2] Delbrück (25) has expressed this view in the following words, "The renewed interest in fundamental research on these agents (i e , bacterial viruses) derives its inspiration principally from the notion that such a study may lead to a better understanding of the nature of *all* types of viruses "

confirm this and demonstrate morphology (Luria, Delbrück, and Anderson, 47) Certain radiations, which inactivate bacteriophages, viruses and bacteria provide us with information regarding their structure Under suitable conditions, a direct inactivating effect due to single ionizations can be obtained with X rays From the data the sensitive volume of the virus particle can be calculated It has been found that the sensitive volume of the few phages which have been studied in this way, is equal or almost equal to the physical volume In other words, nearly every ionizing hit within the physical volume inactivates the phage particle (Luria and Exner, 48) Compare the sensitive volumes of vaccinia virus and *Escherichia coli* which are less than one-half per cent of their total cell volume

Lea and Salaman (43) used α and γ radiations in addition to X rays in a study of vaccinia virus Postulating that the sensitive material is dispersed as a large number of small units, they estimated that each elementary body contains over 900 units The colon bacillus contains a somewhat greater number of sensitive particles These sensitive particles are presumably of many different kinds, for it is unlikely that the destruction of one out of a thousand similar molecules would lead to the death of the cell It may be argued, therefore, that each particle is different and that each is vitally necessary for functioning of the cell Since the organism cannot replace the damaged particle, we seem to be forced to the conclusion that we must regard them as genes, because, if irreplaceable, they must be inherited by duplication during the process of cell division I must confess to a considerable curiosity concerning the results of future comparative studies of an adequate and representative variety of bacteriophage species The observations with vaccinia virus and *E coli* which I have mentioned, would seem to support the hypothesis that the larger mammalian viruses have devolved from bacteria which, acquiring an intracellular habit of growth, have later become obligatory parasites I find just as much difficulty in the extension of this hypothesis to account for the smallest viruses as I find in accepting the alternative hypothesis which postulates that the larger viruses have evolved from the smallest Radiation studies on bacteriophage have been limited to a few species In view of the great diversity of phages it is desirable that such studies should be extended

PHAGE ANTIGEN AND ANTIBODY

Bacteriophage is antigenic and the most important effect of specific antibody upon it is inactivation This inactivation would appear to resemble the neutralization of many mammalian viruses by immune serum As Andrewes and Elford (7) first showed with bacteriophage, virus neutralization generally follows a percentage law This law depends on a dishomogeneity of a virus population as regards susceptibility to inactivation by serum The percentage of virus inactivated in a given time, by a given concentration of serum, is constant over a wide range of virus concentrations Burnet and his co-workers (13) extensively investigated the percentage law, not only for bacteriophages but also for certain mammalian and avian viruses, but if one is to judge by the way in which virus neutralization tests are sometimes reported in the literature, it would seem doubt-

ful whether the significance and implications of the percentage law are universally appreciated If a given virus is neutralized according to the percentage law, the neutralization test should be designed accordingly It is true that the operation of the percentage law may be obscured by the presence of other antibodies, for few if any mammalian viruses appear to be single antigens For example, in order to demonstrate the percentage law with vaccinia virus, it is necessary to use purified, washed elementary bodies and antiserum which has been exhausted of the aggregating LS antibodies by adsorption with killed virus The efficiency of neutralizing antibody varies from species to species of virus Where efficiency is high and the virus is disseminated by the blood stream, the antibody plays an important role in recovery and establishment of immunity and may, as for example in measles, be of prophylactic value Where efficiency is low and the antibody has an inadequate opportunity of attacking the virus while it is vulnerable in transit to new host cells, humoral immunity can, at most, be only of minor importance

There are numerous observations on the neutralization of bacteriophage which suggest that further studies of the mechanisms involved might yield information of interest to workers in the field of mammalian viruses The work of Burnet (10, 12) has shown that bacteriophage particles possess two distinct kinds of receptors which he has designated as A and B The neutralizing antibody combines with the A receptors which may number several hundreds The antibody does not combine with the B receptors which are responsible for the specific adsorption of the bacteriophage particles to the surface of the bacterium, although they may block some of them as a result of spatial overlapping

Tiffany and Rakieten (55) have shown that adsorption of bacteriophage by enterococci is prevented if the bacteria are exposed to immune serum prior to addition of phage More recently Delbrück (23) has pointed out that the inhibitory effect of antibacterial serum can be utilized for rapid titrations of such sera and has suggested that where the effect is highly specific it might be applied to detailed studies of bacterial antigenic structure

Mammalian viruses growing within the host cell are not accessible to the action of immune serum This is true also of bacteriophages, the intracellular growth of which is not affected by exposing the infected bacteria to high concentrations of antiphage serum Under certain conditions an antiserum against the bacterium may be more efficient in inhibiting phage infection and multiplication than antiserum against the phage One is tempted to speculate regarding the bearing of this observation on certain studies of immunity to mammalian viruses which have been interpreted by some as evidence that the immune serum may act on the host cell rather than on the virus

THE BACTERIAL RECEPTORS OF PHAGE

As I have indicated, the surface of a phage particle is of considerable complexity It seems to be a mosaic of two kinds of specifically reacting molecules One is antigenic and reacts with its homologous antibody, the other reacts, somewhat like an antibody, with a corresponding substance on the surface of the sensi-

tive bacterium The latter, the B receptors, are responsible for the first specific step in infection of the cell, namely specific adsorption It is well known that bacteriophage may be inactivated by extracts of bacteria which adsorb it specifically and that the specificity of many phages is related to the antigenic structure of the bacterium Salmonella, dysentery, cholera and staphylococcal phages have been shown to react specifically with a specific surface polysaccharide (*vide* 13, 25, 37, 56) In other instances, specificity may be related to some other kind of antigen, for example, Vi antigen or R antigen or some unidentified substance present on the surface of the cell The inhibiting activity of bacterial extracts is rarely reversible, but Pirie (49) has reported release of phage from heat-killed *Bacillus megatherium* by lysozyme which hydrolyzes a carbohydrate in this organism

Studies of the preferential blocking of the A or B receptors by phage antiserum or phage inhibiting agent present in extracts of the susceptible bacteria, indicate that the A and B receptors are numerous and spatially contiguous A variant dysentery bacteriophage studied by Burnet and Freeman (12) showed a relative insusceptibility to P.I.A , (i e , phage inhibiting agent from dysentery bacilli), a low rate of adsorption by susceptible bacteria, and somewhat increased susceptibility to inactivation by antiserum It may be added that Burnet observed that the activity of B receptors can be readily modified by passage of the phage on a heterologous strain while antigenic character remains unaltered It is tempting to speculate on the possibility that analogous phenomena may occur in the variation of some mammalian viruses

The frequent parallelism between antigenic structure and phage susceptibility has potential applications in antigenic analysis Indeed, Levine and Frisch (45) discovered the existence of suipestifer subgroups as a result of specific phage absorption tests More recently Sievers (53) discovered a new salmonella type (Köln) by phage test, when a bacterium tentatively classified by O and H agglutination tests as *S Breslau* was found to be susceptible to a paratyphoid B phage which did not lyse other Breslau strains The Rakietens (51) observed that organisms of the subtilis group adsorbed and inactivated certain staphylococcal phages and that this was correlated with ability to produce minor agglutinins for strains of staphylococci sensitive to the phage

There is recent evidence that the mechanism of phage adsorption may sometimes require more than the direct interaction of complementary groupings on phage and host cell Anderson (2) has observed that the activity of coli phages T_4 and T_6 on synthetic medium is greatly enhanced by the addition of tryptophane at concentrations as low as 14 μg per ml, and further that tryptophane functions as a specific co-factor in the adsorption of the virus on the host cells Subsequent observations (3) showed that Bz-3-methyl tryptophane, a growth inhibitor, was almost as efficient as tryptophane in promoting adsorption and lysis (4) of ultraviolet-irradiated suspensions of *E coli*

It should be emphasized that in employing bacteriophage as a supplementary tool in antigenic analysis its use should not be restricted to attempts to obtain lysis of bacterial cultures Killed bacterial suspensions or P I.A. preparations

should be employed for adsorption or inactivation of the phage under quantitative conditions With certain bacteriophages such as typhoid Vi phages adsorption is specifically correlated with the presence of Vi antigen but lysis is type or strain specific In this instance specific adsorption of the phage by the living bacterium does not necessarily result in lysis of the organism Further, it may be noted that Burnet and Lush (14), in a study of staphylococcal bacteriophages, observed that certain phages were readily adsorbed by aureus strains which failed to allow multiplication of the phages in broth

Parallelism between antigenic structure and phage susceptibility does not, of course, mean that the antigenic determinant groups and the phage adsorbing or inactivating groups are identical They probably never are The specific Vi phages of *Salmonella typhosa* are adsorbed by bacilli deprived of demonstrable Vi antigen by heat Perhaps, when knowledge of the structure of complex antigens and non-antigenic substances formed by the bacterial cell is further advanced, phage inhibition techniques may prove to be useful analytic tools transcending serological methods, capable of identifying molecular patterns other than those determining antibody specificity

THE SEQUELAE OF PHAGE ADSORPTION

Following adsorption of the phage to the bacterial host cell, there is a latent period during which the phage multiplies With the bacterial cell thus doomed to destruction, the question of whether the bacterial cell may die immediately or survive until lysis occurs, might seem to be of little importance Andrewes and Elford (6), using a very active coli phage of a type inhibited by citrate, were able to demonstrate that on the addition of an excess of phage the organisms were within a few minutes, perhaps seconds, so affected that they could not propagate These authors were unable to demonstrate a similar effect with another bacteriophage, and it is probable that this immediate killing effect may be a property of only a few phages Undoubtedly a considerable surface adsorption of phage is required Since there was no opportunity for the bacteriophage to multiply, it must, on absorption, have blocked some vital process We may speculate on the possibility of some animal viruses and rickettsiae acting in this way

In 1940, Gildemeister and Haagen (38) discovered what is usually described as a toxic substance in the yolk sac of eggs infected with murine typhus rickettsiae The specific lethal effect produced in a few hours, when very high concentrations of murine or epidemic typhus rickettsiae are injected intravenously in the mouse, has been employed extensively in typhus studies during the past war (Rake and Jones (50) have shown that viruses of the lymphogranuloma-psittacosis group produce a lethal or toxic effect which may be analogous) The effect is clearly a property of living, infective rickettsiae, which is lost when the organism loses its infectivity No soluble toxin can be demonstrated

Death of the animal occurs too rapidly to be explained by destruction of susceptible cells as a result of extensive multiplication of rickettsiae within them Mice resistant to infection with strains of the epidemic type die in 1 to 8 hours if a sufficiently heavy dose of infective epidemic rickettsiae is inoculated If we

wish to adopt the hypothesis of a *toxin*, we must postulate one so extremely labile that it cannot survive to exert its effect unless liberated within or on the surface of the susceptible cell

THE INTERFERENCE EFFECT

The immune mechanisms which are operative against bacteria are ineffective against an animal virus resident within a susceptible host cell They may even be relatively inefficient in preventing infection of the cell The host cell, however, can acquire resistance to infection in ways which are attracting increasing attention, because of their importance in virus immunity Invasion of the cell may be blocked if it has been invaded by a variant producing latent infection, or by some other virus related in some way to the first, or even by adsorption of inactivated virus particles It is hardly necessary to stress that these phenomena are of fundamental biological importance Interference has been observed between the following animal viruses non-encephalitogenic and encephalitogenic strains of herpes virus, between Rift Valley fever and pantropic yellow fever virus, Virus III and Shope fibroma virus, between mouse-adapted and monkey strains of poliomyelitis virus, between Eastern and Western equine encephalomyelitis virus, and between influenza A, influenza B and swine influenza virus (*vide* 44 for references) In the instances which I have mentioned the effect was studied in the appropriate laboratory animals or embryonated eggs Andrewes (5), using pneumotropic and neurotropic strains of influenza virus, and Lennette and Koprowski (44) using yellow fever, West Nile, Venezuelan equine encephalomyelitis and influenza A viruses, have studied the interference effect in tissue culture

Undoubtedly, a direct assault on the problems of interference between mammalian viruses will continue to yield information of theoretical and practical value, but the complexities of cellular organization of the mammalian host and the existence of other immunity mechanisms create formidable difficulties In order to elucidate some of the phenomena which may be involved, we must perforce utilize the simplest available materials I do not mean to suggest that observations on interference between bacterial viruses will solve analogous problems with mammalian viruses or that the facts elicited will have any direct bearing or application thereto, but I feel that most will agree that investigation of a *sufficient variety* of bacteriophages will result in the accumulation of useful basic knowledge which will orient and clarify our ideas and provide new suggestions for experimental approach

We may expect that future developments in the prophylaxis of some virus diseases will stem from a better understanding of the interference effect between virus variants and mutants, perhaps developments in treatment as well, as recently reported results with distemperoid virus (40) seem to suggest

I have referred to bacteriophage host cell system as the simplest available It is, unfortunately, far from simple, as the following observations will show Delbrück and Luria (26) advanced the hypothesis of a key enzyme to explain observations on interference between coli phages More recently ingenious

quantitative studies by Delbrück (24) show that this hypothesis is inadequate Delbrück's observations indicate the existence of a "mutual exclusion effect," only the first active phage particle which is adsorbed on the cell membrane being able to establish itself and multiply intracellularly Other phage particles of the same or another species subsequently adsorbed do not multiply Delbrück postulates a change in permeability of the cell membrane which is suddenly established after the first virus particle has penetrated the cell He found that in mixed infections of bacterial populations, the average yield of virus from a bacterium is very much less than in unmixed infections This depressor effect of a second virus, adsorbed presumably on the cell membrane, is difficult to explain It is diminished if antiphage serum is added after the virus has been adsorbed by the bacterium Low concentrations of antibacterial serum may also diminish the depressor action although they do not prevent adsorption of the second virus

BACTERIOPHAGE AS AN AGENT FOR THE CLASSIFICATION AND TYPING OF BACTERIA

Many endeavors have been made, with varying success, to use bacteriophage as an aid in the classification of closely related groups or species of bacteria, or for the differentiation of strains of a given species The literature on the subject is extensive and I shall sketch only the mere outline Undoubtedly the uses of bacteriophage for species and strain identification will be extended as suitable species of phage and appropriate techniques are discovered and independent and concomitant variations of phage and host cell, relevant to the problem, have been elucidated

Conn, Bottcher and Randall (16) have reported that bacteriophage is of value in classifying closely related types of soil bacteria It may be noted that phages for root nodule bacteria seem to be fairly common, and that although it has been suggested that such phages can directly cause legume crop failure, this is doubtful The influence of phage on competition between bacterial strains would seem to require further study

Fisk (33) investigated the lysogenicity of strains of *Staphylococcus aureus* recovered from various human lesions Nineteen out of forty-three coagulase-positive strains were found to be phage carriers, none of these phages was lytic for any of the forty coagulase-negative strains tested None of the coagulase-negative strains yielded a phage lytic for any of the coagulase-positive strains Fisk (34) and Fisk and Mordvin (35) showed that the phages recovered could be applied to identification of particular strains of *Staphylococcus aureus*

Wilson and Atkinson (57) developed a technique of phage typing and extended Fisk's work. They defined twenty-one types The number of types and the comparative rarity of most of them, indicates the possible epidemiological applications of the method Indeed, in four investigations of staphylococcal food poisoning and pemphigus, phage typing was successfully used to trace the source of infection

Keogh, Simmons and Anderson (42) have shown that susceptibility to phage can be used as an auxiliary method in classifying strains of *Corynebacterium*

diphtheriae They found that gravis type I strains carry a phage which lyses gravis type II and intermediate strains Comment on the possibility that phage may play a role in the spontaneous or induced transformations of the diphtheria bacillus seems superfluous

The work of Evans (28, 29, 30) on streptococcal phages is pertinent here and deserves more than this brief reference

Enteric phage typing (21, 18, 31) depends, not on the use of a variety of phages, but on selected mutants of one serological species of specific Vi phage (17) Felix and Callow (32) have extended the principle to the typing of *Salmonella paratyphi B* and *S aertrycke* Twenty-four types and subtypes of *S typhosa* which are epidemiologically stable are now known and it is possible to type at least 95 per cent of Vi strains Time, mercifully perhaps, does not permit an account of the development of the method. I might, however, mention that when Yen and I had differentiated four species of Vi phage (20) it was my intention to attempt to use them in a study of Vi antigen development Phages of types I, III and IV behaved in a reasonable and consistent way but different preparations of type II phage gave discordant results when tested on different strains When it became evident that the strain used for propagation of this phage conditioned its behavior the question of stability of such strain differences arose Therefore we sought to supplement our laboratory observations with epidemiological data I doubt if we would have evolved enteric phage typing if our primary interest had not been an academic one

PHAGE MUTATIONS

Biologically, the most significant feature of bacteriophage is not that it reproduces as a living thing, but that it exhibits discontinuous changes in host affinity that merit the term mutation Phage mutations have been described by Sertic (52), Burnet and Lush (15), Gratia (39), and Luria (46) in which the phage extends its host range by acquiring a capacity to lyse bacterial variants resistant to the parent phage The variation pattern of type II Vi phage is much more complex The range of variants used in typing are type specific When the phage is transferred from one α type of *S typhosa* to another it loses its affinity for the preceding type (19) Differential titrations of such phage preparations strongly suggest that as the phage multiplies the various mutants arise at constant but different rates, ranging from 10^{-4} to 10^{-8} The mutation rate for type E is approximately 10^{-6} Therefore if one desires to obtain phage for type E from phage for type F or phage for any other type, it is necessary to start with a volume and concentration of heterologous phage containing more than 10^{8} phage particles, in order to have at least one phage particle capable of multiplying on cells of type E The mutants of type II Vi phage which are employed for the typing of strains of *S typhosa* have been limited to those which indicate stable and epidemiologically significant differences The acceptance of phage types of *S typhosa* has been based on epidemiological evidence supplemented by laboratory studies on type stability in artificial culture I have recently re-examined my original phage preparations made in 1938, 1939 and the early part of 1940

These remain unchanged except for a slight drop in titre in some instances which represents a loss in activity not greater than 50 per cent The results show not only the satisfactory stability of phage typing preparations but also a stability of the reference type strains of *S typhosa* maintained for the past six to eight years on inspissated egg medium I wish to emphasize the type stability of strains of *S typhosa*, the stability of the phage preparations, and the clear-cut results which may be obtained because of what I am now going to say about other mutations of type II Vi phage and the Vi form of *S typhosa*

Type II Vi phage shows an independent pattern of variation in regard to the size of plaques which it produces The majority of preparations of type II phage employed in typing produce clearings about 0 5 to 0 75 mm in diameter Platings reveal a percentage of distinctly smaller plaques Small plaques which do not owe their smaller size to late development breed true It has proved difficult to recover the normal-plaque variant from a selected small-plaque substrain Platings of the small-plaque variant usually show a few minute plaques barely discernible with the naked eye The minute-plaque variant also breeds true This mutation, which is expressed by a definite and consistent difference in mean plaque diameter, is independent of that mutation in host cell affinity which is the basis of the differentiation of strains of *S typhosa* by Vi phage typing For example, small- and minute-plaque variants of type II Vi phage selected and propagated on type E (classical strain "Ty_2") retain their selective affinity for this type These two independent mutations of type II Vi phage do not, however, exhaust the potential mutation range of this phage

Strains and clones of *S typhosa* vary greatly in their dissociation rates The most obvious dissociation is that which involves the appearance of the non-Vi form or the W form of Kauffmann Other variations occur within the range of Vi forms Some variants merely differ in the duration of the Vi phase, the termination of the Vi phase being correlated with the attainment of a critical population density in a given medium Some differ in colony texture Other differences are revealed when type II Vi phage is propagated on Vi variants stemming from a dissociating strain, these may or may not be correlated with the other differences I have mentioned Such changes in Vi strains are promoted under certain conditions of culture Alkaline drift, which may be pronounced on certain kinds of nutrient agar, particularly when much NaOH has been used for neutralization, appears to be a factor in the appearance and selection of Vi variants which, if used as substrate strains, modify the affinities of type II Vi phage A preliminary study of medium requirements indicates that selected, specific phage preparations are sensitive indicators of the influence of inorganic salts, hydrogen ion concentration and variations in nutriment on rapidly growing cultures of *S typhosa* Specific bacteriophages which might be neglected because of the difficulties of obtaining sufficient and sustained lysis may therefore prove to be useful adjuncts to metabolic studies of the host cell The difficulties which might be expected to arise in the practice of phage typing, because of such Vi variations, can be readily avoided or circumvented Although such changes in the phage and in the host cell are to be avoided in enteric typing, they may pro-

vide interesting and suitable materials for the investigation of virus-host cell relationships under conditions where both can vary

It is frequently stated that phage resistant mutants of bacteria do not adsorb phage which lyses the parent form The Vi phages provide an important exception to this statement which almost seems in danger of becoming dogma The various mutants of type II Vi phage employed in typing are as readily adsorbed by phage-resistant Vi types of *S typhosa* as by the sensitive type which is lysed This is true not only of naturally occurring untypable Vi strains, but also of, strains rendered resistant by the introduction of the cryptic gamma phage of the Croydon type, to which I shall refer later There is no known difference in the Vi antigens formed by the various phage types of *S typhosa* We may conclude, therefore, that all mutants of type II Vi phage must have, in the terminology of Burnet, "the same B receptors" It is obvious that the lytic specificity of the mutants of type II Vi phage depends not on specificity of adsorption but on some specific factor in the host cell which determines penetration and multiplication of the bacteriophage

BIOCHEMICAL STUDIES WITH PHAGE

A number of recent reports suggest the possible usefulness of bacteriophage in studies of bacterial metabolism, provided that certain difficulties can be overcome Spizizen (54) found that colon bacilli suspended in low concentrations of glycine did not multiply but supported phage multiplication Various compounds known to be involved in cellular metabolism enhanced phage multiplication, while metabolic inhibitors prevented multiplication Fitzgerald and Lea (36) have reported that a strain of *E coli*, in acquiring resistances to phosphine GRN became very resistant to a phage lysing the parent strain When ribosenucleic acid was provided this strain became fully sensitive, although naturally occurring phage-resistant mutants remained resistant

Another approach is provided by phage-resistant mutants, obtained by eliminating the sensitive parent form by means of phage Anderson (1) studied mutants of *E coli* obtained in this way and found that 28 out of 57 mutants arising by independent mutations were unable to grow on a synthetic medium They grew abundantly when nutrient broth or minute amounts of yeast extract was added, but systematic tests with amino acids and vitamins failed to yield an active combination

There has been, naturally enough, a tendency to choose for investigation vigorous phages which will lyse the host cell under a wide range of environmental conditions More attention might be paid to species of phage which are apparently weak and capricious, for some might be useful indicators in a study of optimal nutritional and environmental requirements of variants of pathogenic bacteria differing in virulence and ability to produce important antigens Perhaps it might assist progress if bacteriologists, particularly medical and veterinary bacteriologists, thought occasionally of the enzymatic bewilderment of their ill-nourished and ailing victims, struggling for survival in cesspools of their own metabolic products

Conditions which would seem to provide an appropriate environment for colon bacilli are degrading, in more than one sense of the word, for a proud pathogen like the typhoid bacillus

LATENT PHAGES IN S TYPHOSA

Some strains of *S typhosa* are demonstrably lysogenic when the usual techniques are used I mention this kind of lysogenic strain merely for the purpose of differentiating it from another kind of strain that carries a most elusive and interesting phage in a latent state It is estimated that about 10 per cent of strains of *S typhosa* may carry this agent I have provisionally termed such strains "gamma strains" (19) Tests on sensitive strains reveal that cultures of gamma strains containing 1,000,000 organisms per milliliter, rarely contain more than 1,000 lytic phage particles Cultures of greater density contain none and the free agent is presumably re-adsorbed Filtration losses are so serious that it is necessary to depend on centrifugation and heating of the supernatant at 60° for elimination of viable bacteria These gamma phages are somewhat difficult to isolate and difficult to maintain in a state of lytic activity I am inclined to suspect that the lytic agent obtained from "gamma strains" of *S typhosa* does not represent the agent as it occurs naturally in its latent or cryptic state, but that it is a mutant One is very apt to lose this bacteriophage in initial passages Rather, I should say, one appears to lose this phage, because the evidence suggests that it does not die out, but merely returns to its former state of association with the bacterial cell, becoming latent This latency is accompanied by demonstrable changes in the host cell All type D_1 strains which I have examined carry this gamma agent, even strains isolated from the blood Type D_1 was first encountered in the Croydon epidemic of 1937 and has since been encountered frequently in Quebec and elsewhere The gamma agent of D_1 can be isolated as a small-plaque-producing bacteriophage on type A or type E_1 or the non-Vi form of these types If the Vi forms of type A or type E are grown in broth containing an excess of gamma phage, and then plated for colony isolation, Vi colonies are obtained The organisms are resistant to O serum, and are agglutinated by pure Vi serum, but phage tests show that they are resistant to Vi phage which specifically lysed the parent culture Colonies yielding organisms which have become resistant to phage for the original type may represent one or the other of two kinds of Vi type, being either completely resistant to all preparations of type II phage or sensitive only to phage specific for type D_1, the type from which the modifying gamma agent was obtained The "Vi 1" strain of *S typhosa* described by Bhatnagar, Speechly and Singh (19) is characterized by the absence of O and H antigens "Vi 1" is phage type D_1 and, like other D_1 cultures, yields gamma phage I have, on several occasions, found that when the gamma agent from "Vi 1" was used to convert type E to type D_1, that some of the variants obtained were similar to "Vi 1", being not only type D_1, but also lacking H and O antigens Strains infected artificially with gamma agent show a remarkable stability on repeated subculture, yet at any time, the gamma agent may be recovered from them and demonstrated in the way I have

briefly described As far as I know, the gamma agents of *S typhosa* are specific for this species Although a limited type conversion can be achieved under laboratory conditions, there is no epidemiological evidence that such a change can occur with any significant frequency in the spread of typhoid I regret that I am not in a position to throw further light on this phenomenon I have mentioned it for two reasons, first, because it provides an interesting example, a rather special example, of the interference effect Perhaps we may draw some encouragement from the fact that the typhoid bacillus can be vaccinated against a virus disease My second reason for referring to this effect is its possible implications in connection with the antigenic analysis of the salmonella group It has long been recognized that many salmonella strains are lysogenic Some are frankly so, others contain weak phages like the gamma phage of *S typhosa* If the latter can promote serological variation, either directly by infecting and modifying the bacterial cell, or indirectly by eliminating the serologically normal type of bacterial cell and thus permitting emergence of a variant, salmonella phages must be studied systematically in relation to salmonella variation

I have endeavored, in a discursive manner, to indicate some of the ways in which bacteriophage may be usefully employed, either as an aid in bacteriological research or as material for the study of certain virus problems It may be noted that I have avoided several topics I have not attempted to discuss hypotheses of phage or virus multiplication, I have refrained from speculation regarding their origin I have avoided reference to nucleoproteins, transformation of pneumococcal types, and current concepts of the gene Most of us probably feel that what is known about these various subjects represents, so to speak, isolated jig-saw puzzle pieces belonging to the same picture When the missing intervening pieces are found and assembled in place, a map of new territory will be revealed The bacteriologist and virologist will cultivate this new land, applying its resources to human welfare and progress, while the biologist will explore its frontiers in quest of those vital phenomena which, in the words of Claude Bernard, construct "each being according to a pre-existing plan"

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BACTERIOLOGICAL REVIEWS

INDEX

to

Authors and Subjects

Volume 1, 1937 through Volume 10, 1946

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AUTHOR INDEX

SUBJECT INDEX

A

C

D

H

I

Q

R

S

Z